image

AF | 1646

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Welcher *et al.*

Filed: March 29, 2001

For: "CD20/IgE Receptor Like Molecules and Uses Thereof"

Group Art Unit: 1646

Examiner: P. Mertz

Application No. 09/821,821

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on October 10, 2003.

David A. Gass
Registration No. 38,153

SUPPLEMENTAL SUBMISSION IN RESPONSE TO FINAL OFFICE ACTION: DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed herewith is a Declaration pursuant to 37 C.F.R. § 1.132 by Dr. Andrew Welcher, an inventor of the above-named application. The applicants respectfully request that the Declaration, which provides evidence relevant to patentable utility, be considered in conjunction with the applicants' amendment and RCE dated September 21, 2003, filed in response to the final Office Action.

Dated: October 10, 2003

Respectfully submitted,

By
David A. Gass
Registration No.: 38,153

MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Welcher *et al.*

Filed: March 29, 2001

For: "CD20/IgE Receptor Like Molecules and Uses Thereof"

Group Art Unit: 1646

Examiner: P. Mertz

Application No. 09/821,821

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner for Patents, Washington, DC 20231 on _____.

_____
David A. Gass
Registration No. 38,153

DECLARATION OF ANDREW WELCHER, PH.D.,

PURSUANT TO 37 C.F.R. § 1.132

I, Andrew Welcher, do hereby declare and state as follows:

I. Introduction

1. I am a co-inventor of the subject matter claimed in the above-identified U.S. patent application ("the patent application").

2. I am currently employed as a research scientist at Amgen, Inc. in Thousand Oaks, California, where I have worked since 1991. I earned a Bachelor of Science degree in Zoology from Duke University in 1980, a Master degree in Molecular Biophysics and Biochemistry from Yale University in 1985, and a Ph.D. in Molecular Biophysics and Biochemistry from Yale University in 1987. After receiving my Ph.D., I did postdoctoral research with Dr. Eric Shooter in the Department of Neurobiology at Stanford University from 1987 to 1991. I am a named inventor on nine issued U.S.

patents and several other patent applications. A copy of my *curriculum vitae* is attached hereto as Exhibit 1.

3. I have reviewed the patent application, including the pending claims, a copy of which is appended hereto as Exhibit 2.

4. It is my understanding from Amgen's attorneys and from reviewing Patent Office correspondence that the Patent Office has rejected the pending claims in the patent application because the invention is alleged not to have any satisfactory utility. For example, the Patent Office has alleged that the following proposed utilities are unsatisfactory because, allegedly, they are neither specific nor substantial: (a) the use of polynucleotides of the invention "as a tissue specific marker," and (b) the use of the polynucleotides "as a probe or as a chromosomal marker."

5. I make this Declaration to respond to the Patent Office's bases for alleging that the proposed utilities are neither specific nor substantial. By virtue of my education, training, and experience, I believe that I am competent to offer opinions about what average practitioners in my field (who would at least have a doctorate degree and some research experience) would think about the Patent Office's expressed opinions about utility.

II. Utility as a Testicular Tissue Marker

6. Scientists with average experience in the field would generally agree that biological markers that facilitate identification of specific cell or tissue types are valuable for

research and medicine. For example, markers are useful in pathological examination of tissues to identify cell types, identify disease, identify abnormal growth patterns, identify signs of an immune response, identify cancer, and identify cancer metastases. For example, observation of mammary cell markers in liver or bone tissue may facilitate diagnosis of breast cancer metastases into those tissues, or alternatively, abnormal liver or bone function.

7. Exemplary biological molecules that can be useful as tissue-specific markers include: (a) mRNA transcribed from a gene, which can be detected via conventional hybridization procedures with gene-specific probes, to identify cells that express the mRNA; and (b) proteins that can be detected, e.g., with antibodies specific to the protein. Use of polynucleotide sequences in this manner is well established in the literature. *See, e.g.,* Utsunomiya *et al.*, "Cystatin-like metastasis-associated protein mRNA expression in human colorectal cancer is associated with both liver metastasis and patient survival." (*Clin.Cancer Res.*), *8(8)*: 2591-4 (2002)) (*See* Exhibit 3); Nagao *et al.*, "Expression of molecular marker genes in various types of normal tissue: implication for detection of micrometastases." (*Int. Journ. Mol. Med.*), *10(3)*: 307-10 (2001)) (*See* Exhibit 4). Protein molecules are especially useful as markers when the protein is expressed on a cell surface to permit detection without disruption of the cells.

8. An ideal tissue marker, whether mRNA or protein, is strongly expressed only in a single cell or tissue type, without any expression whatsoever in any other cell/tissue in the body. However, there are few absolutes like this in the biomedical field, and such

exquisite tissue specificity of gene expression is no exception. Scientists must generally be content with markers that are predominantly expressed in a single cell or tissue type. If a marker is shown to be predominantly expressed in only one tissue, then detection of a high level of expression in a biological sample normally indicates that the sample contains that tissue. Moreover, when working with markers that are predominantly expressed in a single cell type, a scientist with average skill is capable of adjusting the sensitivity of an assay so that low level expression (in other cell types) goes undetected. In this way, a marker that is "predominantly" express in one tissue behaves as a specific marker for that tissue under the particular assay conditions.

9. Because most genes/proteins that are useful as tissue markers are not expressed exclusively by a single type of cell or tissue, it is desirable to have two or more makers that are predominantly expressed on a single tissue or cell type of interest. The availability of multiple markers for a particular tissue permits identification of the tissue with a greater degree of confidence. There is a continuing need for new markers that are predominantly expressed in a single cell or tissue type.

10. The polynucleotides of the invention are useful for making probes to identify cells of testicular origin expressing this nucleic acid molecule. The patent application contemplates using SEQ ID NO: 1 as a tissue specific probe (e.g., page 41, lines 30-32, "The probes or primers may be used to screen cDNA libraries from various tissue sources believed to express the CD20/IgE-receptor like polypeptide."). More specifically, in Example 3, at page 112, lines 13-18, the patent application discloses

that the DNA sequence from clone agp-96614-al (SEQ ID NO: 1) was utilized as a probe for a Northern tissue expression experiment and the results indicated that "predominant" tissue expression occurred in the testes.[1] This data indicates that polynucleotides of the invention are useful in assays to screen for and identify testicular tissue. A person of ordinary skill in the field would appreciate the use of polynucleotides of the invention for such a purpose from the teachings in the application.

11. The Patent Office has stated that the applicants "have failed to demonstrate the differential expression of the claimed polynucleotide as being expressed only in testicular cells and not in any other tissues. According to Applicants disclosure on page 112, lines 15-18, the instant polynucleotide was also detected in human testes, pancreas, a colon adenocarcinoma cell line and an ovarian carcinoma cell line." While the assay yielded detectable levels of expression in tissue types other than the testes in the assay conditions employed, this does not diminish the significance of the data with respect to the testicular cells, where expression was predominant. There are few absolutes in the biological field, and a person of ordinary skill in the field would readily appreciate that a predominant expression of the claimed sequence in a specific tissue type is sufficient to distinguish that tissue from others in which the sequence is expressed to a lesser degree. Thus, predominance of expression is sufficient to screen for a specific tissue and constitutes a "real world" use of the sequence.[2] For example,

---

[1] This expression profile was independently confirmed in Hulett *et al.*, "Isolation, Tissue Distribution, and Chromosomal Localization of a Novel Testis-Specific Human Four-Transmembrane Gene Related to CD20 and FcepsilonRI-beta." (*Biochem and Biophys. Research Communications 280(1)*:374-379 (2001)) (*See* Exhibit 5). *Compare* page 377, second column and Figure 3 of Hulett *et al.* with Example 3 of the patent application.

[2] As noted above, the Hulett et al. research group subsequently and independently concluded (in their paper) that the expression of the gene corresponding to SEQ ID NO: 1 was testis-specific.

the polynucleotides can themselves be used as probes in assays to detect mRNA corresponding to SEQ ID NO: 1.

12. The protein encoded by the polynucleotide SEQ ID NO: 1 is expressed on the cell surface.[3] As such, practitioners in the field also appreciate that the polynucleotides of the invention (as well as vectors and host cells) can be used to express the encoded protein, which can be used to make antibodies. The antibodies can be used in a variety of standard immunoassays to screen for and identify testicular tissue in a biological sample or *in vivo* in which the protein is expressed. Practitioners in the field consider such utility to be a "real world," practical application. For instance, a pathologist may use tissue-specific antibodies to identify cell types in a biological sample to screen for testicular metastases, or abnormalities in testicular cells.

13. In summary, a polynucleotide that is not exclusively expressed within testicular tissue does not necessarily lack a "real world" application. A predominant expression within testicular tissue is sufficient to make the polynucleotide a useful tissue marker. The fact that testicular specific polynucleotide sequences and proteins may already be known in the field is also irrelevant to the "real world" application of polynucleotide SEQ ID NO: 1. The polynucleotides of the invention can be used with any other existing testicular markers to improve the sensitivity and accuracy of screening for testicular tissue and can help eliminate false positive identifications. Consequently, persons of average skill in the field would agree that the polynucleotide SEQ ID NO: 1 has "real world" use for identifying testis tissue.

---

[3] The protein encoded by the polynucleotide SEQ ID NO: 1 is a four-transmembrane protein.

III. Utility as a Chromosomal Marker

14. Furthermore, the polynucleotide SEQ ID NO: 1 can be used to map the chromosomal location of the CD20/IgE receptor-like gene. The patent application teaches the use of SEQ ID NO: 1 as a chromosomal marker for itself and related genes on page 105, lines 28-33. The use of the sequence as a chromosomal marker has been confirmed in the literature, which has reported that the gene corresponding to polynucleotide SEQ ID NO: 1 and the related CD20, IgE, and HTm_4 genes are clustered within the same region of chromosome 11 (11q 12-13). *See* Hulett *et al.*, at page 378.

15. The Patent Office analogizes the use of polynucleotide SEQ ID NO: 1 as a chromosomal marker to a protein "employed as a molecular weight marker, which is neither a specific or substantial utility." While using a protein as a molecular weight marker or as an analytical standard constitutes a general use that can apply to virtually any protein, the polynucleotide SEQ ID NO: 1 is specifically located in a particular region on a particular human chromosome. There is only one gene at the exact locus on chromosome 11 corresponding to SEQ ID NO: 1, and a limited number of nearby neighbors that map to 11 q12-13. Thus, the polynucleotide of the invention has a highly specific utility as a chromosomal marker, and a person of average experience in this field would find the Patent Office's analogy to a molecular weight marker to be flawed. A molecular weight marker or other analytical standard can be replicated or imitated. However, a chromosomal marker is unique, not of a fungible nature like molecular weight markers or analytical standards. A polynucleotide corresponding to

SEQ ID NO: 1 is useful as a chromosomal marker because it has a unique location and can be used to screen for a specific target: chromosome 11 or the region of chromosome 11, 11q 12-13, where it is located. Practitioners in the field would consider this use to be a "real world" application.

IV. Certification and Oath

16. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date 10/02/03

Andrew Welcher

Dr. Andrew Welcher

Exhibit 1: Curriculum Vitae of Dr. Andrew Welcher

ANDREW AVERY WELCHER

Education

1980	B.S. Zoology Duke University, Durham, North Carolina
1985	M. Phil. Molecular Biophysics and Biochemistry Yale University, New Haven, CT
1987	Ph.D. Molecular Biophysics and Biochemistry Yale University, New Haven, CT

I. Research Experience

2002 -	Research Scientist V Department of Inflammation AMGEN Inc., Thousand Oaks, CA
2000 - 2001	Research Scientist IV Department of Inflammation AMGEN Inc., Thousand Oaks, CA
1999 - 2000	Research Scientist II Department of Inflammation AMGEN Inc., Thousand Oaks, CA
1997 - 1999	Research Scientist II Department of Molecular Genomics AMGEN Inc., Thousand Oaks, CA
1993 - 1997	Research Scientist II Department of Immunology AMGEN Inc., Thousand Oaks, CA
1991 - 1993	Research Scientist I Department of Immunology AMGEN Inc., Thousand Oaks, CA
1987 - 1991	Postdoctoral scholar (with Dr. Eric Shooter) Department of Neurobiology Stanford University, Stanford, CA
1981 - 1987	Dissertation research (with Dr. David Ward) Department of Molecular Biophysics and Biochemistry Yale University, New Haven, Ct.

Teaching Experience

1992	Sole Instructor, UCLA Extension School Principles of Molecular Biology (#420.7; 2 credits)
1981 - 1982	Teaching Assistant, Yale University MB&B 360La/660La MB&B 200 a/Bio 300 a

Patents

2002	Eck receptor ligands, 6,479,250
2000	Eck receptor ligands, 6,087,167
1999	EPH-like receptor protein tyrosine kinases 5,981,245
1999	Nucleic acids encoding EPH-like receptor protein tyrosine kinases 5,981,246
1998	ECK receptor ligands 5,716,934
1998	ECK receptor ligands 5,824,303
1997	Nucleic acids encoding eck receptor ligands 5,650,504
1997	Peripheral myelin protein coding sequence and method 5,599,920
1989	RecA nucleoprotein filament and methods 4,888,274
Filed	EMP-1 coding sequences and method
Filed	Leptin receptor
Filed	Interferon-like protein
Filed	IL-1 RA-like protein
Filed	CD20/IgER related proteins
Filed	Complement regulatory proteins

Awards

1988 - 1990	NIH/NINCDS NRSA 5 F32 NS08443
1990 - 1991	Competitive renewal NIH/NINCDS NRSA 5 32 NS08443

Professional Memberships

1988-	Society for Neuroscience

Community Experience

1993-	California State Science fair judge
1997	Grant Reviewer, Biomedical Research Panel USAMRMC/Neurofibromatosis
1998	Grant Reviewer, Alzheimer's Disease Research Fund California Department of Health Services
1998-	Advisory Committee Neuroscience Research Institute U.C. Santa Barbara

Publications

Giehl, K.M., S. Rohrig, H. Bonatz, M. Gutjahr, B. Leiner, I. Bartke, Q. Yan, L.F. Reichardt, C. Backus, A.A. Welcher, K. Dethleffsen, P. Mestres, and M. Meyer. (2001). Endogenous brain-derived neurotrophic factor abd neurotrophin-3 BDNF and NT-3 antagonistically regulate survival of axotimized corticospinal neurons in vivo. J. Neurosci. 21: 3492 – 3502.

Knott, C., G. Stern, A. Kingsbury, A.A. Welcher, and G.P. Wilkin. An immunohistochemical study of the cellular distribution of neurotrophins and their high affinity receptors in Parkinson's Disease. Submitted.

Nicolson, M., L. Bennett, R.A. Lindberg, T. Bartley, T. Horan, R. Pacifici, B. Karbon, C. Bucher, H.T. Chute, J. Crouse, R. Cupples, B. Guthrie, B. Harpham, J. Marasco, A. Morawiecki, N. Murakami, R. Rosenfeld, and A. A. Welcher. Detection of circulating leptin receptor and complex formation with leptin in human sera. Submitted.

Wahl. R.C., R.Y. Hsu, J. Huff, M.A. Jelinek, K. Chen, P. Courchesne, S.D. Patterson, J.T. Parsons, and A. A. Welcher. (1999). Chicken macrophage stimulating protein is a ligand of the receptor protein-tyrosine kinase Sea. J. Biol. Chem. 274: 26361 – 26368.

Malgrange, B., B. Rogister, P.P. Lefebvre, C. Mazy-Servais, A.A. Welcher, C. Bonnet, R.Y. Hsu, J.M. Rigo, T.R. Van de Water, and G. Moonen. (1998). Expression of growth factors and their receptors in the postnatal rat cochlea. Neurochemical Res. 23: 1133 - 1138.

Rosenfeld, R.D., L. Zeni, A.A. Welcher, L.O. Narhi, C. Hale, J. Marasco, J. Delaney, T. Gleason, J.S. Philo, V. Katta, J. Hui, J. Baumgartner, M. Graham, K.L. Stark, and W. Karbon. (1998). Biochemical, biophysical, and pharmacological characterization of bacterially expressed human agouti-related protein. J. Biochemistry. 37: 16041 - 16052.

Haniu, M., T. Arakawa, E. Bures, Y. Young, J.O. Hui, M.F. Rohde, A.A. Welcher, and T. Horan. (1998). Human leptin receptor: determination of disulfide structure and n-glycosylation sites of the extracellular domain. J. Biol. Chem. 273: 28691 - 28699.

Schweitzer, B., V. Taylor, A.A. Welcher, M. McClelland, and U. Suter. (1998). Neural membrane protein 35 (nmp35): a novel member of a gene family which is highly expressed in the adult nervous system. Mol. Cell. Neuroscience. 11: 260 - 273.

Lindberg, R.A., T.S.C. Juan, A.A. Welcher, Y. Sun, R. Cupples, B. Gutherie, and F.A. Fletcher. (1998). Cloning and characterization of a specific receptor for mouse Oncostatin M. Mol. Cell. Biol. 18: 3357 - 3367.

Kang, H., A.A. Welcher, D. Shelton, and E.M. Schuman. (1997). Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron. 19: 653 - 664.

Raabe, T.D., S. Suy, A.A. Welcher, and G.H. De Vries. (1997). Effect of Neu differentiation factor isoforms on neonatal oligodendrocyte function. J. Neurosci. Res. 50: 755 - 768..

Haniu, M., S. Montestruque, E.J. Bures, J. Talvenheimo, R. Toso, S. Lewis-Sandy, A.A. Welcher, and M.F. Rohde. (1997). Interactions between brain-derived neurotrophic factor and the TRKB receptor. J. Biol. Chem. 272: 25296 - 25303.

Zhou, H., A.A. Welcher, and E.M. Shooter. (1997). BDNF/NT4-5 receptor TrkB and cadherin participate in cell-cell adhesion. J. Neurosci. Res. 49: 281 - 291.

Liu, Y., C.A. Molina, A.A. Welcher, L.D. Longo, and M. De Leon. (1997). Expression of DA11, a neuronal-injury-induced fatty acid binding protein, coincides with axon growth and neuronal differentiation during central nervous system development. J. Neurosci. Res. 48 : 551 -562.

Yan, Q., R.D. Rosenfeld, C.R. Matheson, N. Hawkins, O.T. Lopez, L. Bennett, and A.A. Welcher. (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience. 78: 431 - 448.

Yan, Q., M.J. Radeke, C.R. Matheson, J. Talvenheimo, A.A. Welcher, and S.C. Feinstein. (1997). Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J. Comp. Neurol. 378: 135 - 157.

Cabelli, R.J., K.L. Allendoerfer, M.J. Radeke, A.A. Welcher, S.C. Feinstein, and C.J. Shatz. (1996). Changing patterns of expression and subcellular localization of TrkB in the developing visual system. J. Neurosci. 16: 7965 - 7980.

Lobsiger, C.S., J.P. Magyar, V. Taylor, P. Wulf, A.A. Welcher, Amgen EST Program, and U. Suter. (1996). Identification and characterization of a cDNA and the structural gene encoding the mouse epithelial membrane protein-1. Genomics. 36: 379 - 387.

Barnea, A., N. Aguila-Mansilla. H.T. Chute, and A.A. Welcher. (1996). Comparison of neurotrophin regulation of human and rat neuropeptide Y (NPY) neurons: induction of NPY production in aggregate cultures derived from rat but not human fetal brains. Brain Res. 732: 52-60.

De Leon, M., A.A. Welcher, R.H. Nahin, Y. Liu, M.A. Ruda, E.M. Shooter, and C.A. Molina. (1996). Fatty acid binding protein is induced in neurons of the dorsal root ganglia after peripheral nerve injury. J. Neurosci. Res. 44: 283 - 292.

Taylor, V., A.A. Welcher, Amgen EST Program, and U. Suter (1995). Epithelial membrane protein-1, peripheral myelin protein 22, and lens membrane protein 20 define a novel gene family. J. Biol. Chem. 270: 28824 - 28833.

Snipes, G.J., U. Suter, A.A. Welcher, and E.M. Shooter. (1995). The molecular basis of the neuropathies of mouse and human. Prog. Brain Res. 105: 319 - 325.

Haniu, M., J. Talvenheimo, J. Le, V. Katta, A. Welcher, and M.F. Rohde. (1995). Extracellular domain of neurotrophin receptor trkB: disulfide structure, N-glycosylation sites, and ligand binding. Arch. Biochem. Biophys. 322: 256 - 264.

Rosenfeld, R.D., L. Zeni, M. Haniu, J. Talvenheimo, S.F. Radka, L. Bennett, J.A. Miller, and A.A. Welcher. (1995). Purification and identification of brain-derived neurotrophic factor from human serum. Protein Expression and Purification. 6: 465 - 471.

Fox, G.M., P.L. Holst, H.T. Chute, R.A. Lindberg, A.M. Janssen, R. Basu, and A.A. Welcher. (1995). cDNA cloning and tissue distribution of five human EPH-like receptor protein tyrosine kinases. Oncogene. 10: 897 - 905.

Chang, M., J. McNinch, R. Basu, J. Shutter, R. Hsu, C. Perkins, V. Mar, S. Suggs, A.A. Welcher, L. Li, H. Lu, T. Bartley, P. Hunt, F. Martin, B. Samal, and J. Bogenberger. (1995). Cloning and characterization of the human megakaryocyte growth and development factor (MGDF) gene. J. Biol. Chem. 270: 511 - 514.

Arakawa, T., M. Haniu, L.O. Narhi, J.A. Miller, J. Talvenheimo, J.S. Philo, H.T. Chute, C. Matheson, J. Carnahan, J.C. Louis, Q. Yan, A.A. Welcher, and R. Rosenfeld. (1994). Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. J. Biol. Chem. 269: 27833 - 27839.

Philo, J., J. Talvenheimo, J. Wen, R. Rosenfeld, A.A. Welcher, and T. Arakawa. (1994). Interactions of neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and the NT-3:BDNF heterodimer with the extracellular domains of the TrkB and TrkC receptors. J. Biol. Chem. 269: 27840 - 27846.

Suter, U., G. Jackson Snipes, R. Schoener-Scott, A.A. Welcher, S. Pareek, J.R. Lupski, R.A. Murphy, E.M. Shooter, and P.I. Patel. (1994). Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP22) gene transcripts by two promoters. J. Biol. Chem. 269: 25795 - 25808.

Bartley, T.D., R.W. Hunt, A.A. Welcher, W.J. Boyle, V.P. Parker, R.A. Lindberg, H.S. Lu, A.M. Colombero, R.L. Elliot, B.A. Guthrie, P.L. Holst, J.D. Skrine, R.J. Toso, M. Zhang, E. Fernandez, G. Trail, B. Varnum, Y. Yarden, T. Hunter, G.M. Fox. (1994). B61 is a ligand for the ECK receptor tyrosine kinase. Nature. 368: 558 - 560.

Roa, B.B., C.A. Garcia, U. Suter, D.A. Kulpa, C.A. Wise, J. Mueller, A.A. Welcher, G.J. Snipes, E.M. Shooter, P.I. Patel, and J.R. Lupski. (1993). Charcot-Marie-Tooth disease type 1A. Association with a spontaneous point mutation in the PMP22 gene.N. Engl. J. Med.329: 96 - 101.

Pareek, S., U. Suter, G.J. Snipes, A.A. Welcher, E.M. Shooter, and R.A. Murphy. (1993). Detection and regulation of the peripheral myelin protein PMP-22 in cultured Schwann cells. J. Biol. Chem. 268: 10372 - 10379.

Suter, U., A.A. Welcher, and G. J. Snipes. (1993). Progress in the molecular understanding of hereditary peripheral neuropathies reveals new insights into the the biology of the peripheral nervous system. Trends in Neuroscience. 16: 50 - 55.

Patel, P.I, B.B. Roa, A.A. Welcher, R. Schoener-Scott, B.J. Trask, L. Pentao, G.J. Snipes, C.A. Garcia, U. Francke, E.M. Shooter, J.R. Lupski, and U. Suter. (1992). The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A. Nature Genetics. 1: 159 - 165.

Suter, U., J.J. Moskow, A.A. Welcher, G. J. Snipes, B. Kosaras, R.L. Sidman, A.M. Buchberg, and E.M. Shooter. (1992). A leucine to proline mutation in the putative first transmembrane domain of PMP-22 in the trembler-J mouse. Proc. Natl. Acad. Sci. USA. 89: 4382 - 4386.

Snipes, G. J., U. Suter, A.A. Welcher, and E.M. Shooter. (1992). Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). J. Cell Biol. 117: 225 - 238.

Baldwin, A., C.M. Bitler, A.A. Welcher, and E.M. Shooter. (1992). Studies on the structure and binding properties of the cysteine-rich domain of the rat low affinity nerve growth factor receptor ($p75^{NGFR}$). J. Biol. Chem. 267: 8352 - 8359.

Suter, U., A.A. Welcher, T. Ozcelik, G.J. Snipes, B. Kosaras, U. Francke, S. Billings-Gagliardi, R.L. Sidman, and E.M. Shooter. (1992). Trembler mouse carries a point mutation in a myelin gene. Nature. 356: 241 - 244.

Meakin, S.O., U. Suter, C. Drinkwater, A.A. Welcher, and E.M. Shooter. (1992). The rat trk proto-oncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc. Natl. Acad. Sci. USA. 89: 2374 - 2378.

Welcher, A.A., U. Suter, M. De Leon, G. J. Snipes, and E.M. Shooter. (1991). A myelin protein is encoded by the homologue of a growth arrest specific gene. Proc. Natl. Acad. Sci. USA. 88: 7195 - 7199.

Welcher, A.A., U. Suter, M. De Leon, C.M. Bitler, and E.M. Shooter. (1991). Molecular approaches to nerve regeneration. Phil. Trans. R. Soc. Lond. B. 331: 295 - 301.

De Leon, M., A.A. Welcher, U. Suter, and E.M. Shooter. (1991). Identification of transcriptionally regulated genes after sciatic nerve injury. J. Neurosci. Res. 29: 437 - 448.

Welcher, A. A., C. M. Bitler, M.J. Radeke, and E.M. Shooter. (1991). Nerve growth factor binding domain of the nerve growth factor receptor. Proc. Natl. Acad. Sci. USA. 88: 159 - 163.

Welcher, A.A., A.R. Torres, and D.C. Ward (1986). Selective enrichment of specific DNA, cDNA, and RNA sequences using biotinylated probes, avidin, and copper-chelate agarose. Nuc. Acids Res. 14: 10027 - 10044.

Rigas, B., A.A. Welcher, D.C. Ward, and S.M. Weissman (1986). Rapid plasmid library screening using RecA-coated biotinylated probes. Proc. Natl. Acad. Sci. USA 83: 9591 - 9595.

Book chapters

Wen, J., T. Arakawa, J. Talvenheimo, A.A. Welcher, T. Horan, Y. Kita, J. Tseng, M. Nicolson, and J. S. Philo (1996). A light scattering/size exclusion chromatography method for studying the stoichiometry of a protein-protein complex. In: Techniques in Protein Chemistry VII. Academic Press, Inc. San Diego, CA. pp. 23 - 31.

Welcher, A.A. (1993). Analysis of the structure and function of the fast nerve growth factor receptor. In: Methods in Neuroscience, volume 11: Receptors in Neural Systems. (P.M. Conn. Ed.) Academic Press, Inc. San Diego, CA. pp. 352 - 374.

Welcher, A.A., U. Suter, M. De Leon, G. J. Snipes, S.O. Meakin, and E.M. Shooter. (1992). Isolation of transcriptionally regulated sequences associated with neuronal and non-neuronal cell interactions. In: Progress in Brain Research, volume 94. (A.C.H. Yu et al. eds.) Elsevier Science Publishers, Netherlands pp. 163 - 176.

Bitler, C., S. Meakin, M.J. Radeke, D.L. Shelton, A.A. Welcher, and E.M. Shooter. (1990). The structure and expression of the nerve growth factor receptors. In: Wenner-Gren Int. Symposium series, Brain Repair. Macmillan Press Ltd. pp. 41 - 55.

Recent Abstracts

Rajendra Deshpande, Han Xu, Gregory Dyas, Mei-Mei Tsai, Jill Cai, Heather Inoue, Raj Haldankar, Thomas Horan, Xiaojun Sun, Ching Chen, Timothy Osslund, Felix Shin, Kevin Gaida, Nancy Chow, Sanjay Khare, Andrew Welcher, Karen Sitney, Thomas Boone, Michael Gresser. (2003). Structure-function studies of CTLA-4 binding to B7. American Association of Immunologists Meeting.

Edwards, C.K. III, A.A. Welcher, N.S. Chow, R.F. Baltera, D.M. Miller, D. Rich, M.J. Gresser, C.A. Dinarello, V.A. Marsh, K.A. Storm, D.M. Bankaitis-Davis, and M.P. Bevilacqua. (2001). Selective inhibition of IL-1□ induced proinflammatory gene expression by recombinant IL-1 receptor antagonist (IL-1ra;Anakinara) in human whole blood cultures: contrast to other anti-inflammatory agents. American College of Rheumatology.

Edwards, C.K. III, A.A. Welcher, N.S. Chow, R.F. Baltera, D.M. Miller, D. Rich, M.J. Gresser, C.A. Dinarello, V.A. Marsh, K.A. Storm, D.B. Trollinger, D.M. Bankaitis-Davis, and M.P. Bevilacqua. (2001). Inhibition of proinflammatory gene expression by soluble TNF receptors in human whole blood cultures: marked contrast between TNF p55 receptor (PEG sTNF-R1) and TNF p75 receptor (Etanercept). American College of Rheumatology.

Gutjahr, M., Q. Yan, A.A. Welcher, P. Mestres, M. Meyer, and K.M. Giehl. (1999). A rabbit polyclonal antibody against the extracellular domain of TRKC rescues rat corticospinal neurons from axotomy induced death *in vivo* . Society for Neuroscience.

Lindberg, R.A., T.S.C. Juan, A.A. Welcher, Y. Sun, R. Cupples, B. Guthrie, and F.A. Fletcher. (1998). Identification of the murine oncostatin M receptor uncovers major differences in mouse and human oncostatin M signaling. Tyrosine Phosphorylation and Cell Signaling.

Lindberg, R.A., T.S.C. Juan, A.A. Welcher, Y. Sun, R. Cupples, B. Guthrie, and F.A. Fletcher. (1998). Cloning and characterization of a specific receptor for mouse oncostatin M. 11th International Symposium - Molecular Biology of Hematopoieses.

Karbon, W., R. Rosenfeld, J. Delaney, M. Graham, J. Shutter, J. Marasco, C. Hale, L. Zeni, H. Chute, A. Welcher, and K.L. Stark. (1998). Agrt, a novel agouti-related gene implicated in weight homeostasis. IBC Conference on Obesity.

Kinstler, O.B., Q. Yan, O.T. Lopez, E. Cheung, A.A. Welcher, and C. Le Bel. (1998). N-terminal brain derived neurotrophic factor (BDNF) conjugation to PEG. Third International Symposium on Polymer Therapeutics.

Lindberg, R.A., M. Nicolson, L. Bennett, T. Horan, T. Bartley, R.D. Rosenfeld, R. Pacifici, B. Karbon, M.S. Chang, H.T. Chute, B. Guthrie, B. Harpham, J. Crouse, A.A. Moriawiecki, C. Bucher, N. Murakami, J. Marasco, R. Cupples, and A. A. Welcher. (1997). Generation and use of antibodies to the soluble leptin receptor. North American Association for the Study of Obesity.

Exhibit 2: Claims

WHAT IS CLAIMED

1. (Previously presented) An isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO: 2; and

(c) a nucleotide sequence fully complementary to (a) or (b).

2. - 3. (Canceled)

4. (Previously presented) A vector comprising the nucleic acid of claim 1.

5. (Currently amended) An isolated recombinant host cell comprising the vector of claim 4.

6. (Previously presented) The host cell of claim 5 that is a eukaryotic cell.

7. (Previously presented) The host cell of claim 5 that is a prokaryotic cell.

8. (Previously presented) A process of producing a CD20/IgE-receptor like polypeptide comprising culturing the host cell of claim 5 under suitable conditions to express a CD20/IgE-receptor like polypeptide encoded by the nucleic acid.

9. (Canceled)

10. (Previously presented) The process of claim 8, wherein the vector further comprises a heterologous promoter operatively linked to the nucleotide sequence encoding the CD20/IgE-receptor like polypeptide.

11-50. (Canceled)

51. (Previously presented) A composition comprising a nucleic acid of claim 1 and a pharmaceutically acceptable formulating agent.

52. (Previously presented) A composition of Claim 51 wherein said nucleic acid is contained in a viral vector.

53. (Previously presented) A viral vector comprising a nucleic acid of claim 1.

54. (Previously presented) A fusion polypeptide comprising an amino acid sequence encoded by the nucleic acid sequence of claim 1 fused to a heterologous amino acid sequence.

55. (Previously presented) The fusion polypeptide of claim 54 wherein the heterologous amino acid sequence is an IgG constant domain or fragment thereof.

56-69. (Canceled)

70. (Previously presented) A nucleic acid according to claim 1 attached to a solid support.

71. (Canceled)

72. (Previously presented) The process of claim 8 further comprising isolating the polypeptide from the culture.

Cystatin-like Metastasis-associated Protein mRNA Expression in Human Colorectal Cancer Is Associated with Both Liver Metastasis and Patient Survival[1]

Tohru Utsunomiya,[2] Yoshikazu Hara,[2] Akemi Kataoka, Masashi Morita, Hiroharu Arakawa, Masaki Mori,[3] and Susumu Nishimura

Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838 [T. U., A. K., M. Mori], and Banyu Tsukuba Research Institute in collaboration with Merck Research Laboratories, Ibaraki 300-2611 [Y. H., M. Morita, H. A., S. N.], Japan

ABSTRACT

Purpose and Experimental Design: **We previously reported that an increased expression of cystatin-like metastasis-associated protein (*CMAP*) mRNA is involved in liver-specific metastasis in a mouse model. We also identified its human homologue and showed that the expression of *CMAP* in various human cancer cell lines correlated with the description of malignancy in these cell lines. However, there is still no information available on the clinical significance of *CMAP* expression in human cancer specimens. Thus, we studied the *CMAP* expression levels using a real-time quantitative reverse transcription-PCR for 79 patients with colorectal cancer, including 17 cases with liver metastasis.**

Results: **The mean expression level of *CMAP* in tumor tissue specimens was significantly higher than in the corresponding normal tissue specimens ($P < 0.05$). A higher expression of *CMAP* was significantly correlated with liver metastasis ($P < 0.01$) as well as with a less differentiated histological type ($P < 0.05$) of colorectal cancer. An increased expression of *CMAP* was also identified as the strongest independent factor for liver metastasis based on a multivariate analysis ($P < 0.001$). Furthermore, the prognosis of the patients with a higher expression of *CMAP* was significantly worse than those with a lower expression (5-year survival rate; 49.7% and 75.0%, respectively, $P = 0.038$).**

Conclusions: **These findings imply that the expression level of *CMAP* in human cancer may be a new biomarker for both liver metastasis and the patient's outcome.**

INTRODUCTION

We previously identified a novel metastasis-associated gene with a differential display system in murine carcinoma cells showing a high rate of metastasis to the liver (1). The protein coded by this gene was named CMAP[4] and showed a 22.1–28.1% homology to human family II cystatins. Two other groups have also independently reported the same cDNA sequence of *CMAP* in so-called leukocystatin (2) or cystatin F (3) without identifying the first methionine in the cDNA or noting the relationship of the gene to cancer metastasis.

Cysteine proteinases, such as cathepsin B, H, and L, are regulated by endogenous cysteine proteinase inhibitors named cystatins. The cystatin superfamily is composed of at least four families of closely related proteins, including stefins (family I), cystatins (family II), kininogens (family III), and various structurally related but noninhibitory proteins of family IV (4, 5). In the processes of carcinoma invasion and metastasis, both cysteine proteinases and cystatins have been shown to participate in the dissolution and remodeling of connective tissue and basement membranes (6, 7).

Our previous study (1) revealed that *CMAP* mRNA was selectively overexpressed in all murine liver metastatic tumors but not in any pulmonary metastatic tumors examined. The transfection of *CMAP* antisense DNA into highly metastatic liver cells greatly decreased their *CMAP* expression and metastatic potential, thus indicating that *CMAP* is involved in the liver metastatic ability after the intravasation of malignant cells. The human homologue of *CMAP* was also found in some malignant human cancer, including gastrointestinal cancers by a PCR-based strategy, although the studies were performed with tumor cell lines. For more exact information on the relationship between *CMAP* expression and malignant progression, including metastasis in human cancer, an analysis of surgical or biopsy specimens is required.

This study focused on identifying whether the expression of *CMAP* mRNA is also involved in liver metastasis of human cancer. Interestingly, the *CMAP* expression status in tumor tissue determined by a real-time quantitative RT-PCR showed a strong association with both colorectal liver metastasis and the patient prognosis.

Received 2/11/02; revised 4/29/02; accepted 4/6/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by Grant-in-Aid for Scientific Research (C) 13671311, the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
[2] The first two authors contributed equally to this study.
[3] To whom requests for reprints should be addressed, at Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Tsurumihara 4546, Beppu 874-0838, Japan. Phone: 81-977-27-1650; Fax: 81-977-27-1651; E-mail: mmori@tsurumi.beppu.kyushu-u.ac.jp.

[4] The abbreviations used are: CMAP, cystatin-like metastasis-associated protein; RT-PCR, reverse transcription-PCR.

MATERIALS AND METHODS

Seventy-nine patients with colorectal cancer who underwent surgery at our institutes were entered in this study. The resected tumor and paired nontumor tissue specimens were immediately frozen in liquid nitrogen and kept at −70°C until the extraction of RNA. Written informed consent was obtained from all patients. Among these 79 patients, 17 patients had synchronous hepatic metastases. All patients were closely followed after surgery at regular 1-month intervals. The follow-up ranged from 3 to 63 months with a median of 41 months.

Real-Time Quantitative RT-PCR. Total RNA was extracted from the surgical samples or HL-60 human leukemia cells by the acid-phenol guanidinium method (8, 9). cDNA was synthesized with random hexamer primer and M-MLV reverse transcriptase (Life Technologies, Inc.). The quantitation of the mRNA levels was performed on an ABI Prism 7700 Sequence Detection System (PE Biosystems). Primers and TaqMan probe for human *CMAP* were designed using Primer Express software. To avoid the amplification of contaminating genomic DNA, a TaqMan probe was placed at the junction of exons 3 and 4. To standardize the amount of total RNA added to each reaction, the β-actin mRNA levels were measured as an endogenous control by using TaqMan PDAR Control Reagents. To quantitate the amount of specific mRNA in the samples, a standard curve was generated for each run using five points (20−0.002 ng) of the HL-60 cDNA. In each reaction for tissue samples, 16 ng of cDNA were added in 50 μl of total reaction mixture, and both the *CMAP* and β-actin PCRs were carried out in the same tube in triplicate. The relative expression levels of *CMAP* were obtained by normalizing the amount of *CMAP* mRNA divided by that of β-actin mRNA in each sample (10, 11). The sequences for the TaqMan probes and primers were as follows: sense primer 5′-GTCTGGATGACTGTGACTTCCAAA-3′; antisense primer 5′-AGTGACAACGGAGAACAGGCA-3′; and probe 5′-CAACCACACCTTGAAGCAGACTCTGAGCT-3′.

Statistical Methods. For continuous variables, the data were expressed as the means ± SD. The relationship between the *CMAP* mRNA expression and the clinicopathological factors was analyzed using the χ^2 test and Student's t test. The surviving curves were plotted according to the Kaplan-Meier method, and the generalized Wilcoxon test was applied to compare the survival curve. A multivariate adjustment was also made using a stepwise regression analysis. All tests were analyzed using the StatView software package (Abacus Concepts, Inc.), and the findings were considered significant when $P < 0.05$.

RESULTS

Expression Value of *CMAP* mRNA. We determined the levels of *CMAP* mRNA expression by comparisons with human leukemia cell line HL-60 as the quantifying standard, which expresses human *CMAP* sufficiently. The mean expression level of *CMAP* mRNA in tumor tissue, 0.062 ± 0.074, was significantly higher than 0.043 ± 0.037 in the corresponding normal tissue ($P = 0.042$). The cases with values of less than the mean expression level (0.062) in tumor tissue were considered to be a low expression group ($n = 55$), whereas those with values ≥ 0.062 were considered to be a high expression group ($n = 24$). The clinical implications of *CMAP* expression in patients with colorectal cancer were evaluated by comparisons between these two groups.

Table 1 Clinicopathological data and CMAP mRNA expression in the tumor tissue specimens of 79 patients with colorectal carcinoma

Variables	Low expression[a] ($n = 55$)	High expression[a] ($n = 24$)	P
Sex (male:female)	23:32	10:14	0.990
Age	67.9 ± 9.2	65.6 ± 9.8	0.320
Site			
Colon	32	17	0.281
Rectum	23	7	
Histological grade[b]			
G1	21	3	0.011
G2	34	19	
G3	0	2	
Serosal invasion			
Absent	42	15	0.213
Present	13	9	
Lymph node metastasis			
Absent	29	15	0.419
Present	26	9	
Lymphatic invasion			
Absent	30	15	0.510
Present	25	9	
Venous invasion			
Absent	41	19	0.656
Present	14	5	
Liver metastasis			
Absent	49	13	0.0008
Present	6	11	
Peritoneal dissemination			
Absent	53	23	0.910
Present	2	1	
Duke's classification			
A & B	27	13	0.678
C & D	28	11	

[a] Low and high groups were determined by a mean value of *CMAP* mRNA (0.062).
[b] G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma.

Expression of *CMAP* mRNA and Clinicopathological Characteristics. The clinicopathological factors analyzed are shown in Table 1 in relation to the *CMAP* mRNA expression status. A high expression group showed a significantly higher frequency of moderately or poorly differentiated tumors ($P = 0.011$). Furthermore, the incidence of liver metastasis in the high expression group (11 of 24, 46%) was significantly higher ($P = 0.0008$) than that in the low expression group (6 of 55, 11%). In contrast, other pathological variables such as the serosal invasion, lymph node metastasis, lymphatic invasion, venous invasion, and peritoneal dissemination were not associated with the *CMAP* expression status. Because only 1 patient with lung metastasis was included in this study, the relationship between the *CMAP* expression status and lung metastasis was not considered for a statistical analysis. Table 2 shows the results of a multivariate adjustment with a stepwise regression analysis. In addition to histological type, liver metastasis was selected as the strongest independent factor ($F = 14.22$) for patients in the high *CMAP* expression group ($P < 0.001$).

We also compared the cumulative survival rate of the low expression ($n = 55$) and high expression ($n = 24$) group as

Table 2 Significant variables determined by a stepwise regression model for CMAP expression in the tumor tissue specimens of 79 patients with colorectal carcinoma

Variables	Coefficient	*F*
Histological grade	0.295	8.67
Liver metastasis	0.378	14.22

shown in Fig. 1. The high expression group showed a significantly poorer prognosis ($P = 0.038$) than the low expression group (5-year survival rate; 49.7 and 75.0%, respectively).

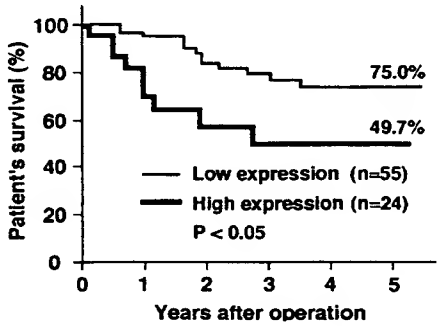

Fig. 1 Comparison of the Kaplan-Meier survival curves in the low expression group ($n = 55$) *versus* the high expression group ($n = 24$). The survival rate for patients in the high expression group was significantly lower than that for patients in the low expression group ($P = 0.038$).

DISCUSSION

We previously reported the identification and cloning of a novel cDNA for a *CMAP* and its close correlation with the liver metastatic potential of cells in the murine system (1). In this study, we tested this correlation in human colorectal cancer, which is the most common human cancer known to develop liver metastasis (12).

In the previous report (1), we analyzed the expression levels of *CMAP* in several organ tissues of mice and found its expression to be detected only in lymphoid organs such as thymus, spleen, and lymph node. Thus, this unique distribution of *CMAP* in lymphoid organs suggested that *CMAP* possesses a physiological association with the immune system. However, there was no information on the *CMAP* mRNA expression in human tissues, including human cancer. We thus determined its expression levels in surgically resected colorectal cancer tissues. Although no *CMAP* expression was observed in the mouse large intestine, *CMAP* expression was detectable in both normal and tumor tissue specimens of human colorectal cancer. One possible explanation for this discrepancy might be attributable to differences in the species studied. Another explanation is the differences in the detection systems for the *CMAP* mRNA expression. In the current study, we used a more sensitive system, a technique of real-time quantitative RT-PCR, to detect its expression levels. The expression of *CMAP* in tumor tissue was found to be markedly up-regulated compared with that of the corresponding normal tissue, thus suggesting the association of *CMAP* expression with the malignant properties of human colorectal cancer.

We therefore evaluated the relationship between the *CMAP* expression status and clinicopathological factors in patients with colorectal cancer. A sharp correlation between the increased *CMAP* expression status and the high frequency of liver metastasis was statistically confirmed by both a univariate and multivariate analysis. Proteinase inhibitors such as cystatins are generally considered to possess antimetastatic activities because proteinases are usually up-regulated and/or abnormally activated in metastatic tumor cells (13–15). We used the differential display method to isolate the cancer-related genes not only in murine cell lines (1) but also in clinical carcinoma specimens (16–18). One such successfully obtained gene was cystatin B (16), the expression of which was drastically suppressed in the tumor tissue of esophageal carcinoma when compared with its corresponding normal tissue. The cystatin B expression demonstrated an inverse relation to lymph node metastasis of the esophageal carcinoma and consequently to the stage of disease. Although the results of this study are consistent with our previous report in the murine system (1), these results appear to be contradictory to the general view of cystatins (13–15), including our previous findings in patients with esophageal cancer (16). However, the production levels of cysteine proteinase inhibitors may possibly increase to compensate for the excessively induced cysteine proteinase in metastatic tumor cells, and as a result, an imbalance may occur between cysteine proteinase and its inhibitor. Such an imbalance seems to play an important role in cancer invasion and metastasis (5, 19).

On the other hand, several studies have also reported a relationship between the activities of proteinase inhibitors and the prognosis of cancer patients, however, the results remain controversial (20–29). In breast (20), lung (21), and head and neck (22) tumors, higher levels of stefin A or stefin B were shown to correlate with a favorable prognosis. On the other hand, the risk of dying has been shown to be significantly higher in patients with increased levels of cysteine proteinase inhibitors in recent studies of breast (23) and colorectal (24) tumors. Furthermore, our results are also consistent with recent reports revealing a correlation between both the increased levels of metalloproteinase inhibitors (25–26) and serine proteinase inhibitors (27–29) and the poor prognosis of cancer patients. For tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1, it has been proposed that besides proteinase inactivation, additional tumorigenic functions may contribute to a worse prognosis, although no such tumorigenic functions were found for *CMAP*. Therefore, the precise function of *CMAP* in the patient prognosis remains unclear. However, *CMAP* may protect producer cells themselves from either excessive proteolysis or from an attack of exogenous proteinases. Alternatively, high levels of *CMAP* may also protect the provisional pericellular matrix of nascent capillaries forming behind the proteinase promoted extensions (27, 29).

In this study, we confirmed the close correlation between the tumor expression levels of *CMAP* and liver metastasis even for human colorectal cancer. Therefore, it is possible that a determination of the *CMAP* mRNA expression of human cancer may help in the identification of patients at high risk for liver

metastasis, and these patients could thereby benefit from careful examinations and extensive treatments for liver metastasis. Recently, the genomic localization of human *CMAP* was determined by fluorescence *in situ* hybridization at our laboratory (30). Additional elucidation of the mechanisms by which the increased *CMAP* expression affects the liver-specific metastatic potential of colorectal cancer is expected to provide a new molecular target for the treatment of liver metastasis.

ACKNOWLEDGMENTS

We thank Toshiko Shimooka, Junko Miyake, and Kazue Ogata for excellent technical assistance.

REFERENCES

1. Morita, M., Yoshiuchi, N., Arakawa, H., and Nishimura, S. CMAP: a novel cystatin-like gene involved in liver metastasis. Cancer Res., *59:* 151–158, 1999.

2. Halfon, S., Ford, J., Foster, J., Dowling, L., Lucian, L., Sterling, M., Xu, Y., Weiss, M., Ikeda, M., Liggett, D., Helms, A., Caux, C., Lebecque, S., Hannum, C., Menon, S., McClanahan, T., Gorman, D., and Zurawski, G. Leukocystatin, a new Class II cystatin expressed selectively by hematopoietic cells. J. Biol. Chem., *273:* 16400–16408, 1998.

3. Ni, J., Fernandez, M. A., Danielsson, L., Chillakuru, R. A., Zhang, J., Grubb, A., Su, J., Gentz, R., and Abrahamson, M. Cystatin F is a glycosylated human low molecular weight cysteine proteinase inhibitor. J. Biol. Chem., *273:* 24797–24804, 1998.

4. Rawling, N. D., and Barrett, A. J. Evolution of proteins of the cystatin superfamily. J. Mol. Evol., *30:* 60–71, 1990.

5. Calkins, C. C., and Sloane, B. F. Mammalian cysteine protease inhibitors: biochemical properties and possible roles in tumor progression. Biol. Chem. Hoppe-Seyler, *376:* 71–80, 1995.

6. Duffy, M. J. Proteases as prognostic markers in cancer. Clin. Cancer Res., *2:* 613–618, 1996.

7. Kos, J., and Lah, T. T. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis, and therapy in cancer. Oncol. Rep., *5:* 1349–1361, 1998.

8. Mori, M., Mimori, K., Inoue, H., Barnard, G. F., Tsuji, K., Nanbara, S., Ueo, H., and Akiyoshi, T. Detection of cancer micrometastasis in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Res., *55:* 3417–3420, 1995.

9. Utsunomiya, T., Inoue, H., Taguchi, K., Shimada, M., Sugimachi, K., and Mori, M. G protein γ7 expression as a new clinicopathological marker in patients with intrahepatic cholangiocarcinoma. Arch. Surg., *137:* 181–185, 2002.

10. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.

11. Gibson, U. E., Heid, C. A., and Williams, P. M. A novel method for real time quantitative RT-PCR. Genome Res., *6:* 995–1001, 1996.

12. Utsunomiya, T., Saitsu, H., Saku, M., Yoshida, K., Matsumata, T., Shimada, M., and Sugimachi, K. Rare occurrence of colorectal cancer metastasis to the liver infected with hepatitis B or C virus. Am. J. Surg., *177:* 279–281, 1999.

13. Lah, T. T., Kokalj-Kunovar, M., Strukelj, B., Pungercar, J., Maganja, D-B., Drobnic-Kosorok, M., Kastelic, L., Babnik, J., Golouh, R., and Turk, V. Stefins and lysosomal cathepsins B, L, and D in human breast carcinoma. Int. J. Cancer, *50:* 36–44, 1992.

14. Navab, R., Mort, J. S., and Brodt, P. Inhibition of carcinoma-cell invasion and liver metastases formation by the cysteine-proteinase inhibitor E-64. Clin. Exp. Metastasis, *15:* 121–129, 1997.

15. Hirai, K., Yokoyama, M., Asano, G., and Tanaka, S. Expression of cathepsin B and cystatin C in human colorectal cancer. Hum. Pathol., *30:* 680–686, 1999.

16. Shiraishi, T., Mori, M., Tanaka, S., Sugimachi, K., and Akiyoshi, T. Identification of cystatin B in human esophageal carcinoma, using differential displays in which the gene expression is related to lymph-node metastasis. Int. J. Cancer, *79:* 175–178, 1998.

17. Shibata, K., Tanaka, S., Shiraishi, T., Kitano, S., and Mori, M. G-protein γ7 is down-regulated in cancers and associated with P 27kip1-induced growth arrest. Cancer Res., *59:* 1096–1101, 1999.

18. Matsuyama, A., Inoue, H., Shibuta, K., Tanaka, Y., Barnard, G. F., Sugimachi, K., and Mori, M. Hepatoma-derived growth factor is associated with reduced sensitivity to irradiation in esophageal cancer. Cancer Res., *61:* 5714–5717, 2001.

19. Knoch, H., Werle, B., Ebert, W., and Spiess, E. Imbalance between cathepsin B and cysteine-proteinase inhibitors is of prognostic significance in human lung cancer. Int. J. Oncol., *5:* 77–85, 1994.

20. Lah, T. T., Kos, J., Blejec, A., Frkovic-Georgio, S., Golouh, R., Vrhovec, I., and Turk, V. The expression of lysosomal proteinases and their inhibitors in breast cancer: possible relationship to prognosis of the disease. Pathol. Oncol. Res., *3:* 89–99, 1997.

21. Ebert, E., Werle, B., Juelke, B., Kopitar-Jerala, N., Kos, J., Lah, T., Abrahamson, M., Spiess, E., and Ebert, W. Expression of cysteine proteinase inhibitor stefin A, stefin B, and cystatin C in human lung tumor tissue. Adv. Exp. Med. Biol., *421:* 259–265, 1997.

22. Budihna, M., Strojan, P., Smid, L., Skrk, J., Vrhvec, I., Rudolf, Z., Zargi, M., Krasovec, M., Svetic, B., Kopitar-Jerala, N., and Kos, J. Prognostic value of cathepsin B, H, L, D and their endogenous inhibitors stefins A and B in head and neck carcinoma. Biol. Chem. Hoppe-Seyler, *377:* 385–390, 1996.

23. Kuopio, T., Kankaanranta, A., Jalava, P., Kroqvist, P., Kotkansalo, T., Weber, E., and Collan, Y. Cysteine proteinase inhibitor cystatin A in breast cancer. Cancer Res., *58:* 432–436, 1998.

24. Kos, J., Krasovec, M., Cimerman, N., Nielsen, H. J., Christensen, I. J., and Brünner, N. Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. Clin. Cancer Res., *6:* 505–511, 2000.

25. Holten-Andersen, M. N., Stephens, R. W., Nielsen, H. J., Murphy, G., Christensen, I. J., Stetler-Stevenson, W., and Brünner, N. High preoperative plasma tissue inhibitors of metalloproteinase-1 levels are associated with shorter survival of patients with colorectal cancer. Clin. Cancer Res., *6:* 4292–4299, 2000.

26. Remacle, A., McCarthy, K., Noël, A., Maguire, T., McDermott, E., O'Higgins, N., Foidart, J. M., and Duffy, M. J. High levels of TIMP-2 correlate with adverse prognosis in breast cancer. Int. J. Cancer, *89:* 118–121, 2000.

27. Grondahl-Hansen, J., Christensen, I. J., Rosenquist, C., Brünner, N., Mouridsen, H. T., Danø, K., and Blichert-Toft, M. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res., *53:* 2513–2521, 1993.

28. Nielsen, H-J., Pappot, H., Christensen, I. J., Brünner, N., Thorlacius-Ussing, O., Moesgaard, F., Danø, K., and Grøndahl-Hansen, J. Association between plasma PAI-1 level and survival in patients with colorectal cancer. Br. Med. J., *316:* 829–830, 1998.

29. Pedersen, A. N., Christensen, I. J., Stephens, R. W., Briand, P., Mouridsen, H. T., Danø, K., and Brünner, N. The complex between urokinase and its type-1 inhibitor in primary breast cancer: relation to survival. Cancer Res., *60:* 6927–6934, 2000.

30. Morita, M., Hara, Y., Tamai, Y., Arakawa, H., and Nishimura, S. Genomic construct and mapping of the gene for CMAP (Leukocystatin/Cystatin F, CST7) and identification of a proximal novel gene, BSCv (C20orf3). Genomics, *67:* 87–91, 2000.

Expression of molecular marker genes in various types of normal tissue: Implication for detection of micrometastases

KUMI NAGAO[1,*], HISASHI HISATOMI[1,*], HIROYUKI HIRATA[1], SHIGEKI YAMAMOTO[1], KAZUMASA HIKIJI[1], MASAHIRO YAMAMOTO[2] and TAICHI KANAMARU[2]

[1]Center for Molecular Biology and Cytogenetics, SRL, Inc., 5-6-50 Shin-machi Hino, Tokyo 191-0002; [2]Department of Surgery, Kobe Rosai Hospital, 4-1-23 Kagoikedori, Chuo-ku, Kobe 651-0053, Japan

Received April 19, 2002; Accepted May 29, 2002

Abstract. Many researchers have investigated the expressions of candidates for a suitable reverse transcription nested polymerase chain reaction (RT-PCR) marker. But typically biomarkers often have false-positive results. We assessed whether epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA) and prostate-specific membrane antigen (PSM) could be detected in 28 different types of normal human sources. Using RT-nested PCR assay, EGFR mRNA was also detected in various types of normal tissue, including pancreas, prostate and uterus. CEA was detected in various types of normal tissue, including prostate, uterus, bladder and spleen. PSM mRNA was also detected in various types of normal tissue, including kidney, liver, skeletal muscle, spleen, bladder and ovary. We report here that the expression of these biomarkers in normal cells might have induced false-positives, and that further enhancement of sensitivity might compromise specificity. Conversely, these biomarkers can be utilized for attempts to define micrometastases in various types of tumors whose cells express these tissue-specific genes.

Introduction

Attempts to find cancer cells in circulatory blood or lymph nodes using present PCR techniques have been made in various types of malignant tumors, including breast, colon and prostate cancers. Epidermal growth factor receptor (EGFR) and carcinoembryonic antigen (CEA) are tactile molecular targets for the detection of circulatory cancer cells in patients with breast and colon cancers, respectively (1-4). Prostate-specific membrane antigen (PSM) is not specific to malignant neoplasms of the prostate, but detection of prostate cancer cells in circulatory blood has effectively been realized by the RT-PCR method due to its high specificity for prostatic epithelia (5). In the breast cancer field, histological detection of auxiliary lymph node metastasis is still the most valuable prognostic parameter, but about 30% of node-negative patients relapse within five years, suggesting that current methods are inadequate for identifying metastatic disease (6). Therefore, more sensitive, PCR-based methods for the detection of metastatic cells have become available, enabling the amplification of cancer cell-specific EGFR messages by RT-PCR assay (3,4). However, false-positive findings were recorded in a percentage of patients with benign disease (4). CEA mRNA, a typical metastasis biomarker, was detected frequently in peripheral blood from normal volunteers, when systemic blood was sampled by venesection needle (7). Namely, needle-cored epithelial cells produced positive results for detecting hematogenous micrometastasis.

An ideal tumor marker, consistently expressed in tumor samples and not at all in normal lymph node, normal peripheral blood and normal tissues remains to be identified. Although, detection of biomarker-positive cells in circulatory blood or lymph nodes has effectively been realized owing to their high specificity for the tumorous cells, unfortunately, almost tumor biomarkers were detected in not only tumor tissues but also in various types of normal tissue. For example, Jonas *et al* (1) described that CEA mRNA can be detected in cutaneous epithelial cells, and Smith *et al* (8) also described that prostate specific antigen, a typical marker for prostate cancer, mRNA can be detected in a variety of non-prostate cells.

It seems that circulatory tumor cells are present when molecular marker-positive cells are detected in blood samples, because tumor cells have a high ability for propagation and reproduction. These abilities induce the release of tumor cells into systemic blood or lymph nodes. That is, it seems that the existence of the releasing of normal organic cells in peripheral blood or lymph nodes is a rare event. If this hypothesis is true where did the detected EGFR or CEA-positive cells in normal blood come from? Normal mucous cells have more ability for reproduction than tumor cells, indicating that it is easy to explain the existence of the release of normal organic cells. Since there is a great need for more data for the expression of these biomarkers in various types of normal organic cells, we have clarified which organic cells expressed EGFR, CEA and PSM messages.

Correspondence to: Dr Hisashi Hisatomi, Center for Molecular Biology and Cytogenetics, SRL, Inc., 5-6-50 Shin-machi Hino, Tokyo 191-0002, Japan
E-mail: hisatomi@srl-inc.co.jp

*Contributed equally

Key words: epidermal growth factor receptor, carcinoembryonic antigen, prostate-specific membrane antigen, metastases

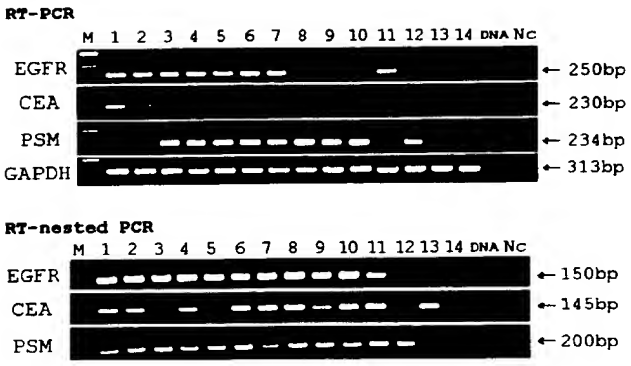

Figure 1. Detection of mRNA for EGFR, CEA and PSM, and representative results of RT-nested PCR analysis. Lanes 1-7, Human Total RNAs: lane 1, bone marrow; lane 2, colon; lane 3, heart; lane 4, kidney; lane 5, liver; lane 6, lung; lane 7, prostate; lane 8, spleen; lane 9, stomach; lane 10, testis. Lanes 11-13, Marathon-Ready™ cDNA: lane 11, breast; lane 12, ovary; lane 13, lymph node; lane 14, leukocyte. DNA, genomic DNA; Nc, negative control (RNase free water); M, molecular weight marker.

Materials and methods

Extracted total RNA, 22 different types of Human Total RNA, from normal samples were purchased (Clontech, CA, USA), including adrenal gland, bone marrow, whole brain, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid gland and uterus. Synthesized cDNA, 6 different types of Marathon-Ready™ cDNA, from normal samples were purchased (Clontech), including bladder, breast, cerebral cortex, leukocyte, lymph node and ovary. cDNA was synthesized with a random primer (Invitrogen, CA, USA) using 1.0 μg of each human total RNA.

The primer set for amplification of an EGFR mRNA was designed according to Genbank U48722, using as outer primers exon 1: 5'-AGCTCTTCGGGGAGCAGCGATGCG-3' and exon 2: 5'-GGAAAGATCATAATTCCTCTGCACATA-3' and using inner primers exon 1: 5'-TGCTGGCTGCGCTCTGC-3' and exon 2: 5'-GACCACCTCACAGTTATTGAACAT-3' (150 bp).

The primer set for amplification of a CEA mRNA was designed according to Genbank M29540, using outer primers exon 8: 5'-GGACCTATGCCTGTTTTGTCT-3' and exon 10: 5'-GTTGCAAATGCTTTAAGGAAGAAG-3' and using inner primers exon 9: 5'-TTCTCCTGGTCTCTCAGCTGG-3' and exon 10: 5'-GTTGCAAATGCTTTAAGGAAGAAG-3' (145 bp).

The primer set for amplification of a PSM mRNA was designed according to Genbank M99487, using forward primer exon 1: 5'-TCTTTCTCCTCGGCTTCCTCTTCG-3' and outer reverse primer exon 3: 5'-GCTCAACAGAATCCAGGC CAAATT-3'. The second PCR reaction mixture was prepared using forward primer and inner reverse primer: 5'-GGATTG AATTTGCTTTGCAAGCTG-3' (200 bp).

The primer set for amplification of a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, as an internal control, was designed according to Genbank M33197, using primers exon 6-7: 5'-AACAGCCTCAAGATCATCAGCAA-3' and exon 8: 5'-TCAGGTCCACCACTGACACGTT-3' (313 bp).

For the template, 2 μl cDNA (equivalent 0.2 μg total RNA) was used in the first round of PCR and 2.5 μl of PCR product was used in the second round of PCR with AmpliTaq Gold DNA polymerase (PE Applied Biosystems, CA, USA). Each PCR reaction was made at 35 cycles (95°C for 30 sec, 60°C for 40 sec, 72°C for 30 sec). The final elongation step was 72°C for 5 min. As a negative control, target cDNA was substituted by nuclease free water, indicating that contamination was prevented in these assays. Half of the PCR product was separated using electrophoresis in a TBE buffer on 3.0% agarose gel, stained with ethidium bromide, and detected with ultraviolet light (Figs. 1 and 2).

The PCR products of EGFR, CEA and PSM were purified using a High pure PCR product purification kit (Roche Molecular Biochemicals diagnostic, IN, USA) and were directly sequenced using the BigDye terminator Cycle Sequencing ready Reaction kit (PE Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems). The sequence was finally compared with the target mRNA sequence.

Results

Each PCR mixture presented a single band on electrophoresis. The sizes of the PCR products obtained with EGFR were 150 bp, with CEA 145 bp, while those obtained with PSM were 200 bp (Fig. 1). The sequences of 150, 145, and 200 bp PCR products were in complete agreement with the EGFR, CEA, PSM mRNA sequences, respectively. These detection systems have about 100 copies/μg total RNA of sensitivity (Fig. 2). The positive control amplification for G3PDH

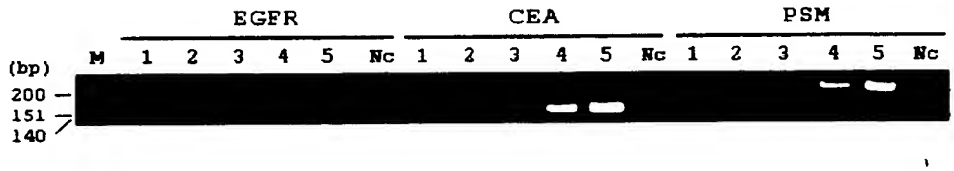

Figure 2. Dilution experiment. EGFR, CEA and PSM transcripts were synthesized from pGEX-4T plasmids (Pharmacia Biotech, Uppsala, Sweden) by *in vitro* transcription with T7 polymerase. Lane 1-5, serially diluted transcripts: lane 1, 25 copy; lane 2, 50 copies, lane 3, 75 copies, lane 4, 100 copies, lane 5, 200 copies/assay. The mRNA could be detected in 100 copies/assay by RT-nested PCR. M, molecular weight marker.

Table I. Detection of EGFR, CEA and PSM mRNA in various types of normal tissue.

	EGFR		CEA		PSM	
	RT-PCR	Nested PCR	RT-PCR	Nested PCR	RT-PCR	Nested PCR
Adrenal gland	+	+	-	+	+	+
Bladder	+	+	+	+	+	+
Bone marrow	+	+	+	+	-	+
Brain	+	+	-	+	+	+
Breast	+	+	+	+	-	+
Cerebral cortex	+	+	-	-	+	+
Cerebellum	+	+	-	-	+	+
Colon	+	+	+	+	-	+
Fetal brain	+	+	-	+	+	+
Fetal liver	+	+	-	+	+	+
Heart	+	+	-	-	+	+
Kidney	+	+	-	+	+	+
Leukocyte	-	-	-	-	-	-
Liver	+	+	-	-	+	+
Lung	+	+	+	+	+	+
Lymph node	-	-	-	+	-	-
Ovary	-	-	-	-	+	+
Pancreas	+	+	-	-	-	+
Placenta	+	+	-	+	+	+
Prostate	+	+	+	+	+	+
Skeletal muscle	-	+	-	+	+	+
Small intestine	-	+	+	+	+	+
Spleen	-	+	-	+	+	+
Stomach	-	+	+	+	+	+
Testis	-	+	+	+	+	+
Thymus	-	+	-	+	+	+
Thyroid	-	+	+	+	+	+
Uterus	-	+	+	+	+	+

afforded a single band of 312 bp, and its message was positive in all samples. No signals at 145, 150 and 200 bp were seen in the water controls, which were negative (Fig. 1).

EGFR mRNA was detected in adrenal gland, bladder, bone marrow, whole brain, breast, cerebral cortex, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid gland and uterus, but not detected in leukocyte, lymph node and ovary (Table I). CEA mRNA was detected in adrenal gland, bladder, bone marrow, whole brain, breast, colon, fetal brain, fetal liver, kidney, lung, lymph node, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid gland and uterus, but not detected in cerebellum, cerebral cortex, heart, liver, leukocyte, ovary and pancreas (Table I). PSM mRNA was detected in adrenal gland, bladder, bone marrow, whole brain, breast, cerebral cortex, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid gland and uterus, but not detected in leukocyte and lymph node (Table I). Identical experiments were performed 3 times to confirmed reproducibility (data not shown).

Discussion

RT-nested PCR detection of circulating cancer cells is susceptible to laboratory variation and contamination (7). In our laboratory, the risk of aerosols including PCR products yielding contamination was prevented by an air conditioner and the partitioning of six separate rooms. The six air-conditioned rooms are for 1, extraction; 2, preparation of cDNA and PCR mixture; 3, cDNA synthesis; 4, first PCR; 5, second PCR; and 6, electrophoresis room. From 1-5 the rooms were set up with high pressure air conditioning, the number 6 room was set up with low pressure air conditioning. With these high quality assays, three typical molecular markers showed bands of expression in several normal organic cells. We believe these are not the results of contamination because: a) it is not another targeted mRNA because the primers sufficiently differ from other mRNA sequences, and sequencing of the PCR products of each mRNA confirms the sequence is of these genes; b) omitting the template (water control) yields no bands; therefore, contaminations are not present.

It has been suggested that the molecular identification of cancer cells in circulation may be useful in predicting the presence of micrometastasis in several cancer types. EGFR and CEA have been generally believed to be expressed only by malignant cells. If this were true of both mRNA, then detecting both mRNA in cells outside a neoplasmic organ would indicate metastasis. Although CEA mRNA was not detected in normal leukocytes, its message has been reported as positive in normal cutaneous epithelial cells (7). PSM mRNA have also been reported to be detected in normal brain, heart, liver, lung, kidney, spleen, and thyroid gland (9). We report here that normal organic cells may express low levels of EGFR, CEA and PSM mRNA detectable by nested primer PCR. We unexpectedly found these mRNA in non-tumorous sources. On the other hand, appreciable percentages of false-positive cases with EGFR, CEA and PSM biomarkers have been frequently reported (10-12). In addition, Wharton *et al* (7) recommend that >29 PCR cycles were avoided for detecting circulating CEA-positive cells. False-positively EGFR findings were recorded in 25% of patients with benign diseases (4). It is important that a molecular biomarker is introduced as a sensitive assay, RT-nested PCR method is essential for good biomarkers. However, the risk of a highly sensitive method yielding false-positives must be prevented. Our data point out that further enhancement of sensitivity may compromise specificity. Although, it is a rare event that normal organic cells spread in blood or lymph nodes, the possibility exists that normal releasing cells induced false-positive results. The data of Wharton *et al* (7) on CEA similarly confirms the low yield of positive signals with CEA PCR. This must be considered when RT-nested PCR screening for these markers is performed as a diagnostic measure in blood from cancerous patients. EGFR, CEA and PSM mRNA-PCR may ultimately be clinically useful; however, as with most

medical testing, sensitivity and specificity will not be absolute and will need to be balanced.

Our data confirm that nested PCR is a highly sensitive method of detection of these mRNA. Unfortunately, it appears that low levels of these mRNA are present in a variety of normal organic cells. However, most importantly, EGFR, CEA and PSM mRNA were not detected in normal leukocytes. That is, these markers were not malignancy specific, but rather were tissue-specific. Even if expressions of these markers were truly tissue-specific, prior reports with sensitive PCR methods have found promiscuous transcription of other so-called tissue specific genes (13,14). Hence, our findings of these mRNA in non-malignant cells should not be so surprising. These data are particularly important in that tissue-specific expression of tumor markers could have a significant impact. For example, because CEA mRNA was detected in colon, kidney, lung, pancreas, prostate, spleen, testis, thymus, thyroid gland and uterus, a first mRNA could be utilized to find CEA-positive cells in hematogenous spreads from the neoplasm of these organs. Perhaps changing the concept of a time-dependent spread of cancer cells via an extension through the capsule, spread to regional lymph nodes or peripheral blood, and then to distant sites. Tumor cells are often released in CEA-negative sources, including peripheral blood and lymph nodes, it seems that CEA-positive cells are tumor cells. We, and others (3,4,11), have developed a sensitive PCR method for detection of EGFR mRNA and have evaluated it for the detection of circulating micrometastases in the blood of breast, lung (15), and colon (16) carcinoma patients. However, these results have not been given for tumor-specific expression, because its mRNA was detected in normal breast, lung and colon cells. Therefore, EGFR mRNA may be useful for detecting micrometastatic cells in pancreas, prostate, uterus and bladder cancer, because its mRNA was detected in normal pancreas, prostate, uterus and bladder cells, respectively. CEA mRNA may be a useful marker for micrometastases in prostate, uterus, bladder and spleen cancer as well as lung (17), thyroid gland (18), gastric (19), breast (20) and colorectal (21) cancer, and the possibility of amplifying them by PCR provides a powerful tool to analyze pathological transcripts of any tissue-specific genes by using any accessible cell.

Our results indicate the need for caution in interpreting positive PCR results for these mRNA, and this may be generally applicable to what are believed to be tissue-specific genes.

Acknowledgements

We are grateful to Koh-ichi Matsumoto (SRL Inc, Kobe) for his support.

References

1. Jonas S, Windeatt SAOB, Fordt C, *et al:* Identification of carcinoembryonic antigen-producing cells circulating in the blood of patients with colorectial carcinoma by reverse transcriptase polymerase chain reaction. Gut 39: 717-721, 1996.
2. Nakanishi H, Kodera Y, Torii A, *et al:* Detection of carcinoembryonic antigen-expressing free tumor cells in peritoneal washes from patients with gastric carcinoma by polymerase chain reaction. Jpn J Cancer Res 88: 687-692, 1997.
3. Falette NS, Artagaveytia N, Rostan MC, *et al:* Analysis of epidermal growth factor receptor mRNA expression by polymerase chain reaction assay in 94 human breast adenocarcinoma tumors. Breast Cancer Res Treat 30: 275-282, 1994.
4. Leitzel K, Lieu B, Curley E, *et al:* Detection of cancer cells in peripheral blood of breast cancer patients using reverse transcription polymerase chain reaction for epidermal growth factor receptor. Clin Cancer Res 4: 3037-3043, 1998.
5. Noguchi M, Miyajima J and Noda S: Detection of circulating tumor cells in patients with prostate cancer using prosate specific membrane-derived primers in the polymerase chain reaction. Int J Urol 4: 374-379, 1997.
6. Beckmann MW, Niederacher D, Massenkeil G, *et al:* Expression analyses of epidermal growth factor receptor and HER-2/neu: no advantage of prediction of recurrentce or survival in breast cancer patients. Oncology 53: 441-447, 1996.
7. Wharton RQ, Patel H, Joans SK, *et al:* Venesection needle coring increases positive results with RT-PCR for detection of circulating cells expressing CEA mRNA. Clin Exp Metastasis 18: 291-294, 2000.
8. Smith MR, Biggar S and Hussain M: Prostate-specific antigen messenger RNA is expressed in non-prostate cells: implications for detection of metastases. Cancer Res 55: 2640-2644, 1995.
9. Renneberg H, Friedetzky A, Konrad L, *et al:* Prostate specific membrane antigen (PSM) is expressed in various human tissues: implication for the use of PSM reverse transcription polymerase chain reaction to detect hematogenous prostate cancer spread. Urol Res 27: 23-27, 1999.
10. Piva MG, Navaglia F, Basso D, *et al:* CEA mRNA identification in peripheral blood is feasible for colorectal, but not for gastric or pancreatic cancer staging. Oncology 59: 323-328, 2000.
11. De Luca A, Pignata S, Casamassimi A, *et al:* Detection of circulating tumor cells in carcinoma patients by a novel epidermal growth factor receptor reverse transcription-PCR assay. Clin Cancer Res 6: 1439-1444, 2000.
12. Israeli RS, Miller WH, Su SL, *et al:* Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostate tumor cells: comparisoin of prostate-specific membrane antigen and prostate-specific antigen-based assay. Cancer Res 54: 6306-6310, 1994.
13. Chelly J, Voncordet JP, Kaplan JC, *et al:* Illegotomate transcription: trnascription of any gene in any cell type. Proc Natl Acad Sci USA 86: 2617-2621, 1989.
14. Sakar G and Sommer SS: Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science 244: 331-334, 1989.
15. Damstrup L, Rude Voldborg B, Spang-Thomsen M, *et al: In vitro* invasion of small-cell lung cancer cell lines correlates with expression of epidermal growth factor receptor. Br J Cancer 78: 631-640, 1998.
16. Rajagopal S, Huang S, Moskal TL, *et al:* Epidermal growth factor expression in human colon and colon carcinomas: antisense epidermal growth factor receptor RNA down-regulates the proliferation of human colon cancer cells. Int J Cancer 62: 661-667, 1995.
17. Perey L, Benhattar J, Peters R, *et al:* High tumour contamination of leukaphereses in patients with small cell carcinoma of the lung: a comparison of immunocytochemistry and RT-PCR. Br J Cancer 85: 1713-1721, 2001.
18. Bojunga J, Dragan C, Schumm-Draeger PM, *et al:* Circulating calcitonin and carcinoembryonic antigen m-RNA detected by RT-PCR as tumour markers in medullary thyroid carcinoma. Br J Cancer 85: 1546-1550, 2001.
19. Okada Y, Fujiwara Y, Tamamoto H, *et al:* Genetic detection of lymph node micrometases in patients with gastric carcinoma by multiple-marker reverse transcriptasr-polymerase chain reaction assay. Cancer 92: 2056-2064, 2001.
20. Marchetti A, Buttitta F, Bertacca G, *et al:* mRNA markers of breast cancer nodal metastases: comparison between mmamaglobin and carcinoembryonic antigen in 248 patients. J Pathol 195: 186-190, 2001.
21. Sadahiro S, Suzuki T, Tokunaga N, *et al:* Detection of tumor cells in the portal and peripheral blood of patients with colorectial carcinoma using competitive reverse transcriptase polymerase chain reaction. Cancer 92: 1251-1258, 2001.

Biochemical and Biophysical Research Communications 280, 374–379 (2001)
doi:10.1006/bbrc.2000.4088, available online at http://www.idealibrary.com on IDEAL®

Isolation, Tissue Distribution, and Chromosomal Localization of a Novel Testis-Specific Human Four-Transmembrane Gene Related to CD20 and FcεRI-β

Mark D. Hulett,*,[1] Eloisa Pagler,* June R. Hornby,* P. Mark Hogarth,† Helen J. Eyre,‡ Elizabeth Baker,‡ Joanna Crawford,‡ Grant R. Sutherland,‡ Stephen J. Ohms,§ and Christopher R. Parish*

*Division of Immunology and Cell Biology and §Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, ANU, P.O. Box 334, Canberra, ACT 2601, Australia; †Austin Research Institute, Austin and Repatriation Medical Centre, Melbourne, Victoria 2084, Australia; and ‡Centre for Medical Genetics, Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide, South Australia 5006, Australia

Received November 27, 2000

CD20 and the β subunit of the high affinity receptor for IgE (FcεRIβ) are related four-transmembrane molecules that are expressed on the surface of hematopoietic cells and play crucial roles in signal transduction. Herein, we report the identification and characterization of a human gene, *TETM4*, that encodes a novel four-transmembrane protein related to CD20 and FcεRIβ. The predicted TETM4 protein is 200 amino acids and contains four putative transmembrane regions, N- and C-terminal cytoplasmic domains, and three inter-transmembrane loop regions. TETM4 shows 31.0 and 23.2% overall identity with CD20 and FcεRIβ respectively, with the highest identity in the transmembrane regions, whereas the N- and C-termini and inter-transmembrane loops are more divergent. Northern blot and RT-PCR analysis suggest that TETM4 mRNA has a highly restricted tissue distribution, being expressed selectively in the testis. Using fluorescence *in situ* hybridization and radiation hybrid analysis, the *TETM4* gene has been localized to chromosome 11q12. The genes for CD20 and FcεRIβ have also been mapped to the same region of chromosome 11 (11q12-13.1), suggesting that these genes have volved by duplication to form a family of four-transmembrane genes. TETM4 is the first nonhematopoietic member of the CD20/FcεRIβ family, and like its hematopoietic-specific relatives, it may be involved in signal transduction as a component of a multimeric r c ptor complex. © 2001 Academic Press

Key Words: four-transmembrane; TM4SF; tetraspanin; CD20; FcεRIβ; testis; gene localization; chromosom 11; signal transduction.

CD20, the β subunit of the high affinity receptor for IgE, and HTm_4, comprise a family of related proteins that contain four membrane spanning regions. All three proteins are expressed specifically in hematopoietic cells; CD20 on B cells (1), FcεRIβ on mast cells and basophils (2), and HTm_4 on cells of myeloid and lymphoid origin (3). Both CD20 and FcεRIβ have been well characterized as playing important roles in initiating signal transduction events as components of multimeric receptor complexes. CD20 has been shown to have the capacity to regulate B cell proliferation and differentiation as part of a large cell surface complex with MHC-I, MHC-II, CD40, and the tetraspanins CD53, 81 and 82 (1, 4). FcεRIβ is a key component of the tetrameric $\alpha\beta\gamma_2$ FcεRI complex on mast cells and basophils, and plays a crucial role in enhancing cell surface expression of the complex and amplifying signal transduction events mediated upon the interaction of receptor-bound IgE with multivalent allergen (2, 5, 6). The functional role of HTm_4 is unknown, but as for CD20 and FcεRIβ, it is likely to contribute to the signalling of a multimeric receptor complex. In this study, we report the isolation, tissue distribution and chromosomal localization of a human gene that encodes a novel member of the CD20/FcεRIβ/HTm_4 family.

MATERIALS AND METHODS

Isolation of RNA and first strand cDNA synthesis Total cellular RNA was prepared by homogenising 100 mg of tissue in 1 ml of Trizol reagent (Gibco-BRL, Grand Island, NY), upon which the aqueous fraction was recovered and RNA precipitated using isopropanol. First strand cDNA was produced from 5 μg of total RNA by priming with an oligo dT primer (NotdT, 5′ AACTGGAAGAATTCGCGGCCGCAGGAAT[18] 3′) using a First Strand cDNA synthesis system (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

[1] To whom correspondence should be addressed. Fax: 61-2-6249-2595. E-mail: mark.hulett@anu.edu.au.

0006-291X/01 $35.00

PCR and nucleotide sequence analysis. PCR was performed on 10 ng of first strand cDNA in the presence of 100 ng of each oligonucleotide primer, 1.25 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD) for 35 amplification cycles. 3'-RACE was performed by PCR as described above with the oligonucleotide primer TET-1 (5'-GTCATCTCCTTTCAAATTATCAC-3', hybridizes to nucleotides 24–46 of the TETM4 cDNA) and the NotdT primer (see above). Nucleotide sequencing was performed by direct sequencing of amplified cDNA fragments using an Applied Biosystems 377 sequencer.

Northern blot analysis. Northern analysis of multiple human tissue blots (Clonetech, Palo Alto, CA) was performed by probing membranes with the full length TETM4 cDNA, labelled by random priming (Megaprime DNA labelling system; Amersham, Buckinghamshire, UK), using Expresshyb solution (Clonetech, Palo Alto, CA) as specified by the manufacturers. Membranes were washed in 1× SSC for 40 min at room temperature followed by 0.1× SSC for 40 min at 60°C and exposed to X-ray film.

Southern blot analysis. 10 μg of genomic DNA was digested with a range of restriction enzymes, separated on a 1% agarose gel, and transferred to a Hybond-N nylon filter (Amersham, Buckinghamshire, UK). The blot was probed with the full length TETM4 cDNA labelled by random priming and hybridized in a 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's solution and 100 μg/ml salmon sperm DNA at 42°C. The membrane was washed in 1× SSC for 40 min at room temperature followed by 0.1× SSC for 40 min at 65°C and exposed to X-ray film.

Fluorescence in situ hybridization. A 1100-bp genomic fragment of the *TETM4* gene, produced by PCR amplification with oligonucleotide primers TET-2 (5'-TTCAACTCAAAGCCCCTTGC-3', hybridizes to nucleotides 155–174 of the TETM4 cDNA) and TET-4 (5'-CCTTGGATATGGTTTTAACAAAG-3', nucleotides 290–268), was nick-translated with biotin-14-dATP and hybridized *in situ* at a final concentration of 15 ng/ml to metaphases from two normal males. The fluorescence *in situ* hybridization (FISH) method was as previously described (7), with the exception that chromosomes were stained before analysis with both propidium iodide (as counterstain) and diaminophenylindole (DAPI) (for chromosome identification). Images of metaphase preparations were captured by a cooled CCD camera using the chromoScan image collection and enhancement system (Applied Imaging Int. Ltd.).

Radiation hybrid analysis. The *TETM4* gene was mapped using the medium resolution Stanford G3 panel of 83 clones. Screening of the panel was performed by PCR amplification of a 1100-bp *TETM4*-specific fragment using oligonucleotide primers TET-2 and TET-4 (see above). Amplifications were performed on 10 ng of each sample DNA under the above conditions.

RESULTS AND DISCUSSION

Identification and Isolation of the TETM4 cDNA

In order to investigate the possible existence of additional novel members of the CD20/FcεRIβ/HTm_4 family, the human dbEST (public expressed sequence tags, GenBank database) was searched with a consensus peptide sequence corresponding to a conserved region of the second transmembrane (TM) region of the three known human family members (GYPFWGAIFFSISG) (3, 8, 9). A number of ESTs were identified, all from testis libraries (GenBank Accession Nos. AI149899, AA416972, AA411806, AA707529, AA470059, AA436088, AA781801, AI002083, AA435988), which contained a region homologous to the conserved search peptide. Sequence analysis of the ESTs suggested that they were fragments of a single gene which was related to, yet distinct from, CD20/FcεRIβ/HTm_4. The EST sequences were assembled into a single contig of 695 bp, and examination of the compiled sequence, suggested an open reading frame that encoded for a putative protein of 200 amino acids. An oligonucleotide primer was designed to the predicted 5' untranslated region of the cDNA (TET-1, 5'-GTCATCTCCTTTCAAATTATCAC-3', hybridizes to nucleotides 24-46 of the TETM4 cDNA) and used in 3' rapid amplification of cDNA ends (RACE)-PCR with the oligo-dT primer NotdT (see Materials and Methods), on first-strand cDNA generated from human testis total RNA. A product of 707 bp was amplified, that upon direct sequencing, was determined to encode the complete coding region predicted from the EST contig, confirming that the cDNA sequence was derived from a single mRNA. The cDNA was also cloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA), and multiple clones were analysed, which revealed an identical sequence to that determined from direct sequencing of the PCR product. The nucleotide and deduced amino acid sequence encoded by the full length cDNA, designated TETM4 (for testis expressed transmembrane-4, see below), is shown in Fig. 1.

The complete TETM4 cDNA is 695 bp long and contains a canonical polyadenylation signal sequence at nucleotides 669–673 (Fig. 1). The cDNA encodes for a deduced protein of 200 amino acids with a predicted molecular weight of 22.2 kDa. Hydropathy analysis indicates the presence of four hydrophobic regions that represent four putative transmembrane domains. Using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html), which predicts membrane spanning regions and their orientation, TETM4 is predicted to have four strong transmembrane helices which are likely to adopt a membrane topology with both the N- and C-termini intracellular. On the basis of this prediction, the TETM4 protein can be divided into the following domains; four transmembrane domains (TM-1, TM-2, TM-3 and TM-4) of 22, 21, 20 and 22 amino acids respectively, N- and C-terminal cytoplasmic domains of 48 and 18 amino acids, respectively, two extracellular loops of 14 and 22 amino acids and a short intracellular loop of 13 amino acids (Fig. 1). Significantly, both CD20 and FcεRIβ have been shown experimentally to have a topology on the cell surface as predicted here for TETM4 (1, 2). However, it should be noted that it remains possible that TETM4 may adopt an alternate topology with both the N- and C-termini extracellular. Furthermore, it also needs to be considered that TETM4 may not be expressed on the cell surface but instead is localized on a subcellular membrane(s).

```
-56      GACTAGACTGAAGTACCAACTAAGTCATCTCCTTTCAAATTATCACCGACACCATC

  1  ATGGATTCAAGCACCGCACACAGTCCGGTGTTTCTGGTATTTCCTCCAGAAATCACTGCT
  1  M  D  S  S  T  A  H  S  P  V  F  L  V  F  P  P  E  I  T  A

 61  TCAGAATATGAGTCCACAGAACTTTCAGCCACGACCTTTTCAACTCAAAGCCCCTTGCAA
 21  S  E  Y  E  S  T  E  L  S  A  T  T  F  S  T  Q  S  P  L  Q

121  AAATTATTTGCTAGAAAAATGAAAATCTTAGGGACTATCCAGATCCTGTTTGGAATTATG
 41  K  L  F  A  R  K  M  K  I  L  G  T  I  Q  I  L  F  G  I  M

181  ACCTTTTCTTTTGGAGTTATCTTCCTTTTCACTTTGTTAAAACCATATCCAAGGTTTCCC
 61  T  F  S  F  G  V  I  F  L  F  T  L  L  K  P  Y  P  R  F  P

241  TTTATATTTCTTTCAGGATATCCATTCTGGGGCTCTGTTTTGTTCATTAATTCTGGAGCC
 81  F  I  F  L  S  G  Y  P  F  W  G  S  V  L  F  I  N  S  G  A

301  TTCCTAATTGCAGTGAAAAGAAAAACCACAGAAACTCTGATAATATTGAGCCGAATAATG
101  F  L  I  A  V  K  R  K  T  T  E  T  L  I  I  L  S  R  I  M

361  AATTTTCTTAGTGCCCTGGGAGCAATAGCTGGAATCATTCTCCTCACATTTGGTTTCATC
121  N  F  L  S  A  L  G  A  I  A  G  I  I  L  L  T  F  G  F  I

421  CTAGATCAAAACTACATTTGTGGTTATTCTCACCAAAATAGTCAGTGTAAGGCTGTTACT
141  L  D  Q  N  Y  I  C  G  Y  S  H  Q  N  S  Q  C  K  A  V  T

481  GTCCTGTTCTTGGGAATTTTGATTACATTGATGACTTTCAGCATTATTGAATTATTCATT
161  V  L  F  L  G  I  L  I  T  L  M  T  F  S  I  I  E  L  F  I

541  TCTCTGCCTTTCTCAATTTTGGGGTGCCACTCAGAGGATTGTGATTGTGAACAATGTTGT
181  S  L  P  F  S  I  L  G  C  H  S  E  D  C  D  C  E  Q  C  C

601  TGACTAGCACTGTGAGAATAAAGATGTGTTAAAATAAAAAA
```

FIG. 1. Nucleotide and deduced amino acid sequence of human TETM4. The nucleotide sequence is numbered with the first nucleotide of the translational initiation codon as +1. The amino acid sequence is presented below the nucleotide sequence in single letter code, with the four putative transmembrane regions underlined. The predicted initiation codon and stop codon are in bold type and the polyadenylation signal sequence is underlined. GenBank Accession No. AF321127.

Analysis of the TETM4 Amino Acid Sequence

The alignment of the predicted amino acid sequence of TETM4 to that of human CD20 (8), FcεRIβ (9), and HTm_4 (3), indicates an overall identity of 31.0% (55.4% similarity), 23.2% (47.0%) and 26.4% (51.9%), respectively (Fig. 2). The identity of TETM4 to CD20/FcεRIβ/HTm_4 is highest in the transmembrane regions, with the N- and C-termini and intra-transmembrane loop regions showing little homology. TETM4 contains a number of charged/polar residues in its TM regions, including a glutamine residue (Q54) in the first TM domain, an asparagine in each of the second (N97) and third (N121) TM domains, and a glutamic acid (E177) in the forth TM domain. All of these residues, with the exception of N97, are also conserved in CD20/FcεRIβ/HTm_4 (Fig. 2). Interestingly, charged/polar residues are also common in the transmembrane regions of other multi-membrane spanning proteins such as the tetraspanins, which like CD20 and FcεRIβ, associate with other membrane molecules (10). Other interesting structural features of the TETM4 protein include two cysteine residues in its second extracellular loop region (C147 and C156), which are also found in CD20/FcεRIβ/HTm_4, and may be involved in forming an intra- or inter-chain disulphide bond(s). The C-terminal cytoplasmic tail of TETM4 contains a cluster of five cysteine residues in a 12 residue stretch (C189, 194, 196, 199 and 200), which may also be involved in disulphide bond formation.

Identification of a TETM4 Splice Variant

The PCR amplification of the TETM4 cDNA with oligonucleotide primers TET-1 and NotdT (see Materials and Methods) from human testis also led to the isolation of a putative splice variant. Nucleotide sequence analysis indicated that this cDNA is 542 bp long and is identical to the TETM4 cDNA, however, it is missing the coding region for the third trans-

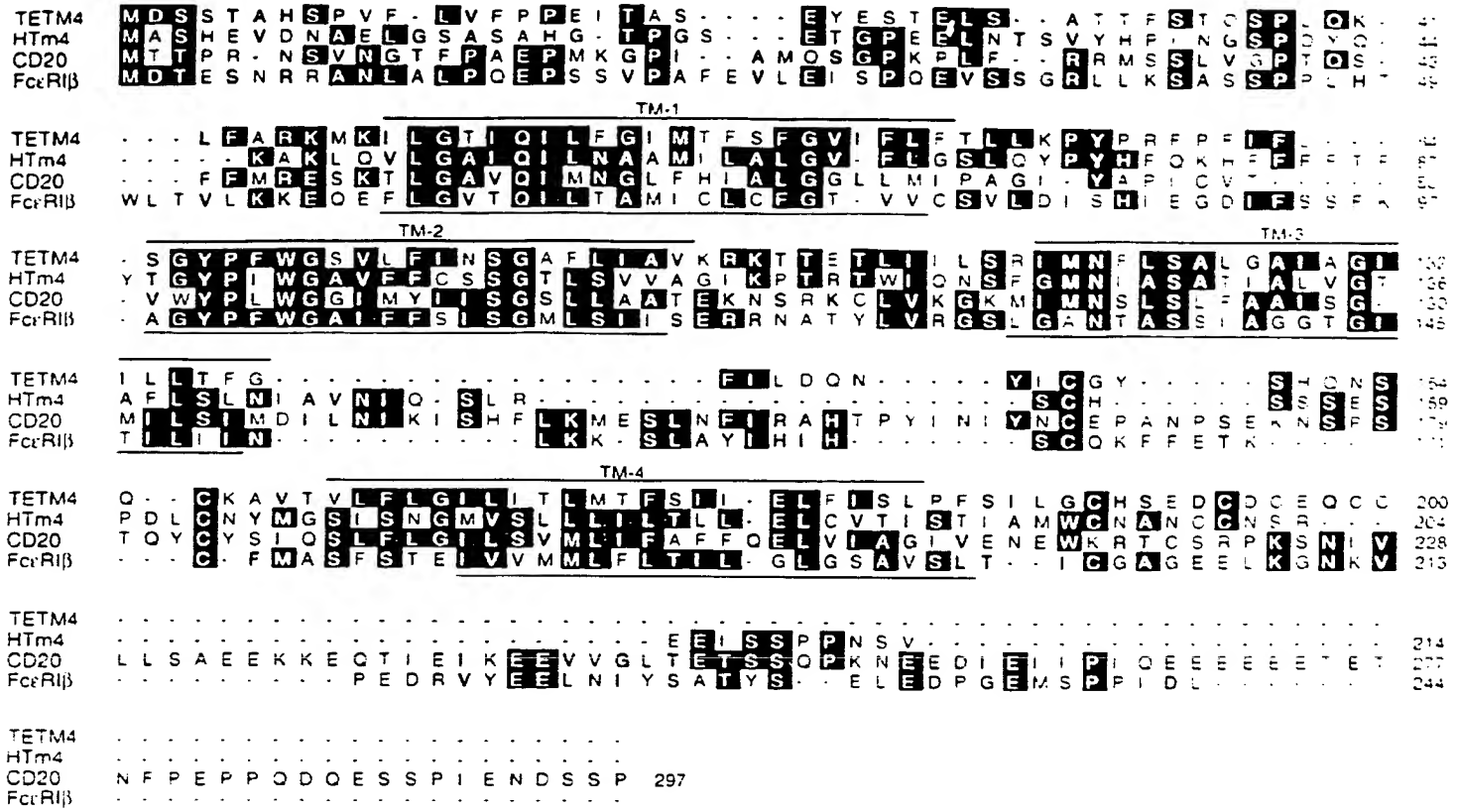

FIG. 2. Amino acid alignment of TETM4 with CD20, FcεRIβ and HTm$_4$. The amino acid sequences are presented in single letter code. The alignment was performed using CLUSTALW (GCG package) and adjusted manually. Gaps (−) have been introduced to maximise alignment of the sequences. Identical or similar residues between at least two sequences are shaded in black or grey, respectively. Similar residues are defined as: D, E (acidic); A, G, I, L, V (aliphatic); N, Q (side-chain containing amide group); F, W, Y (aromatic); R, H, K (basic); S, T (side-chain containing hydroxyl group). The positions of the four putative transmembrane regions for TETM4 and FcεRIβ are overlined and underlined, respectively. The predicted TM regions for HTm$_4$ and CD20 are very similar to that shown for FcεRIβ, with the exception that CD20 contains a continuous hydrophobic stretch between TM-1 and TM-2. The GenBank Accession Nos. are: TETM4, AF321127; CD20, AAA35581; FcεRIβ, AAA60269; HTm4, AAA62319.

membrane and second extracellular domains (nucleotides 394 to 547 encoding amino acids L113 to F163). This splice variant is also represented in the EST database by two clones (GenBank Accession Nos. AA411806 and AA781801). The deduced polypeptide encoded by this cDNA would contain only three transmembrane regions, and would therefore be predicted to have a membrane topology with the C-terminal domain extracellular, as opposed to intracellular for the four-transmembrane form. A putative TETM4 protein with this different topology would be likely to have an altered function. The lack of the fourth transmembrane region may influence possible association(s) with other membrane molecules, and the shifting of the C-terminal domain from intracellular to extracellular may change any potential signalling capacity mediated through interactions with intracellular signalling proteins. Clearly, it would be of interest to determine if this variant encodes for a functional protein.

Tissue Distribution of TETM4 mRNA

The tissue distribution of TETM4 mRNA was investigated by Northern blot analysis of a range of human tissues. A strong band centered around 0.7 kb was detected only in testis and not in spleen, thymus, prostate, ovary, small intestine, colon, peripheral blood leukocyte, heart, brain, placenta, liver, lung, skeletal muscle, kidney or pancreas (Fig. 3). Prolonged exposure of the Northern blot failed to reveal any significant signal in any tissue other than testis. Reverse-transcriptase (RT)-PCR analysis of TETM4 mRNA was also performed on first strand cDNA made from mRNA isolated from the above range of human tissues. Amplification with oligonucleotides TET-2 (see Materials and Methods) and TET-3 (5′-CAGTAACAGCCTTA-GACTGAC-3′, hybridizes to nucleotides 537–516 of the TETM4 cDNA) produced the expected product of 383 bp only in testis, but not any other tissue (data not shown). These data suggest that TETM4 shows an

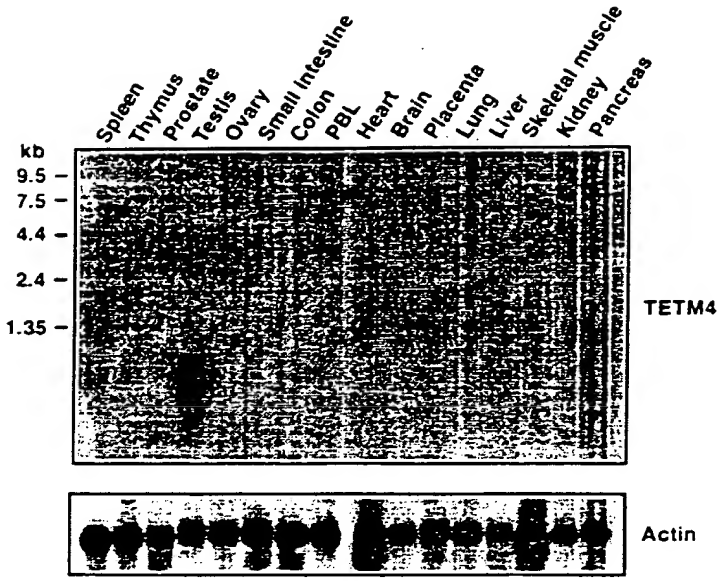

FIG. 3. Northern blot analysis of TETM4 mRNA expression. Multiple tissue Northern blot filters (Clonetech, Palo Alto, CA) were hybridized with ^{32}P-labelled full-length TETM4 cDNA in ExpressHyb solution (Clonetech, Palo Alto, CA) as specified by the manufacturer. The filters were rehybridized with a control ^{32}P β-actin cDNA and show approximately equal amounts of mRNA loaded per lane; heart and skeletal muscle have two β-actin transcripts. The positions of molecular weight makers (in kilobases) are indicated. Exposure times were 12 h for both TETM4 and β-actin.

extremely specific tissue distribution, being found only in the testis. The identification of TETM4 ESTs derived only from human testis libraries, also supports the proposed testis specific expression. Thus, in contrast to CD20/FcϵRIβ/HTm$_4$, which are all hematopoietic specific, TETM4 is the first member of this family that is expressed in a non-hematopoietic tissue.

Southern Blot Analysis and Chromosomal Localization of the TETM4 Gene

Southern blot analysis was performed on restricted human genomic DNA using the full length TETM4 cDNA as a probe. A simple banding pattern was produced that is consistent with the *TETM4* gene being a single copy gene (Fig. 4). To determine the chromosome localization of the *TETM4* gene, fluorescence *in situ* hybridization (FISH) was performed on metaphase chromosomes of two normal males using a 1100-bp *TETM4*-specific genomic fragment as a probe. Twenty metaphases from the first male were examined for a fluorescent signal, which was present in all 20 metaphases in the region 11q12-11q13, with 57% of the signal located in the central portion of band 11q12 (Fig. 5). Similar results were obtained from hybridizations of the probe to metaphases from the second normal male. Radiation hybrid mapping of the *TETM4* gene was also performed using the medium resolution Stanford G3 panel of 83 clones. Screening of the panel by PCR amplification of a 1100 bp *TETM4*-specific fragment using oligonucleotide primers TET-2 and TET-4 (see Materials and Methods), indicated that *TETM4* is most closely associated with the Stanford Human Genome Centre marker SHGC-20674, with a LOD score of 13.23 (data not shown). SHGC-20674 is not ordered on a Stanford map; however, Stanford has linked it to marker SHGC-35409 which is ordered on the Stanford Radiation Hybrid Map. The markers most closely associated with *TETM4* are flanked by markers D11S335 and D11S4363. Searches of the Cytogenetic Yac Bank (http://sgiweb.ncbi.nlm.nih.gov/Zjing/yac.html) placed the flanking markers D11S1335 and D11S4363 on Yac WC11.5 which spans the 11q12 cytogenetic band. The genes for CD20, FcϵRIβ and HTm$_4$ have also been mapped to the same region of chromosome 11 (11q12-13) (3, 11, 12). These data suggest that the TETM4, CD20, FcϵRIβ, and HTm$_4$ genes have evolved by duplication and divergence of the same ancestral gene to form a family of four-transmembrane genes.

The tetraspanins comprise a distinct family of four-transmembrane molecules that are expressed on both hematopoietic and non-hematopoietic cells. In contrast to the CD20/FcϵRIβ/HTm$_4$/TETM4 family, the tetraspanins appear to form a far more extensive family and are found in species ranging from schistosomes to humans. At least 20 members have been described in

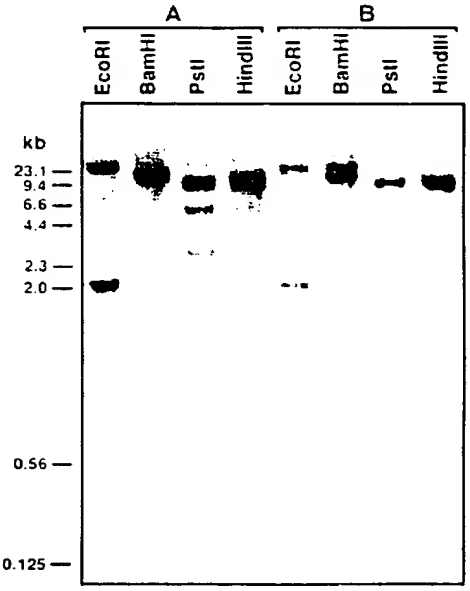

FIG. 4. Southern blot analysis of the *TETM4* gene. 10 μg of human genomic DNA was isolated from two male individuals (A and (B), restricted with a range of enzymes, and Southern analysis performed by hybridizing with random primed [^{32}P]-labelled TETM4 cDNA in 50% formamide, 6 × SSC, 0.5% SDS and 5 × Denhardt's solution. The blot was washed under high stringency conditions and exposed to X-ray film. The positions of molecular weight makers (in kilobases) are indicated.

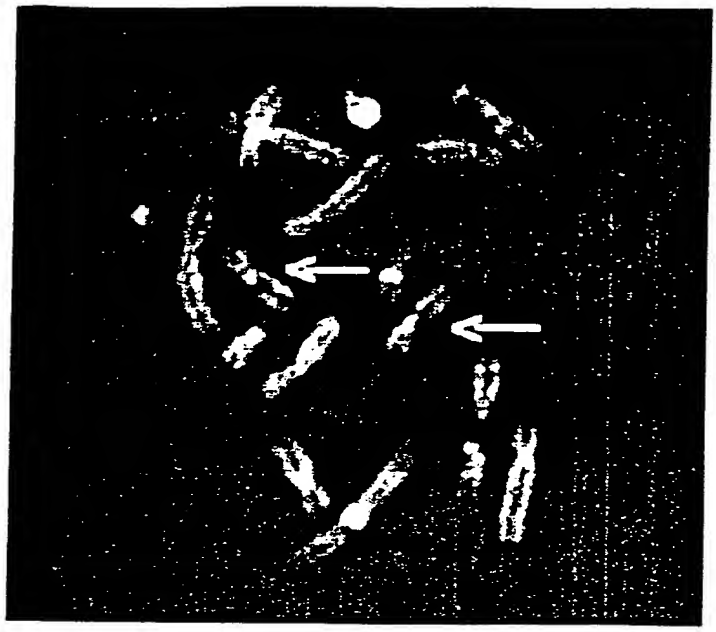

FIG. 5. Chromosomal localization of the *TETM4* gene by FISH. Partial metaphases are displayed showing FISH with a TETM4 probe. Normal male chromosomes have been counterstained with DAPI. Hydridization sites on chromosome 11 are indicated by arrows. FISH signals and the DAPI banding pattern have been merged.

the human, including CD9, CD37, CD53, CD81, and CD82 (10). It is possible that like the tetraspanins, the CD20/FcεRIβ/HTm$_4$/TETM4 family may be much larger. Indeed, we have recently identified a number of additional family members that we are currently characterising (M. Hulett, manuscript in preparation).

The testis-specific expression of TETM4 raises some intriguing questions as to its function. As described above, CD20 and FcεRIβ are expressed specifically on hematopoietic cells where they form components of multimeric cell surface receptor complexes, and play important roles in signal transduction (1, 5, 6). It is therefore tempting to speculate that TETM4 may also associate with receptor complexes on the surface of specific cells in the testis and participate in signalling events. Clearly, to address these possibilities and to delineate the function of TETM4, further fundamental issues need to be addressed such as determining the cellular and subcellular distribution of TETM4 in the testis. These studies are currently in progress.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia and the Wellcome Trust.

REFERENCES

1. Tedder, T. F., and Engel, P. (1994) CD20: A regulator of cell-cycle progression of B lymphocytes. *Immunol. Today* **15**, 450–454.
2. Kinet, J.-P. (1999) The high affinity IgE receptor (FcεRI): From physiology to pathology. *Annu. Rev. Immunol.* **17**, 973–976.
3. Adra, C. N., Lelias, J. M., Kobayashi, H., Kaghad, M., Morrison, P., Rowley, J. D., and Lim, B. (1994) Cloning of the cDNA for a hematopoietic cell-specific protein related to CD20 and the β subunit of the high affinity IgE receptor: Evidence for a family of proteins with four membrane-spanning regions. *Proc. Natl. Acad. Sci. USA* **91**, 10178–10812.
4. Szollosi, J., Horejsi, V., Bene, L., Angelisove, P., and Damjanovich, S. (1996) Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. *J. Immunol.* **157**, 2939–2946.
5. Lin, S., Cicala, C., Scharenberg, A. M., and Kinet, J.-P. (1996) The FcεRI β subunit functions as an amplifier of FcεRI γ-mediated cell activation signals. *Cell* **85**, 985–995.
6. Donnadieu, E., Jouvin, M.-H., and Kinet, J.-P. (2000) A second amplifier function for the allergy-associated FcεRI -β subunit. *Immunity* **12**, 515–523.
7. Callen, D. F., Baker, E., Eyre, H. J., Chernos, J. E., Bell, J. A., Sutherland, G. R. (1990) Reassessment of two apparent deletions of chromosome 16p to an ins(11;16) and a t(1;16) by chromosome painting. *Ann. Genet.* **33**, 219–221.
8. Tedder, T. F., Streuli, M., Schlossman, S. F., and Saito, H. (1988) Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes *Proc. Natl. Acad. Sci. USA* **85**, 208–212.
9. Kuster, H., Zhang, L., Brini, A. T., MacGlashan, W. J., and Kinet, J.-P. (1992) The gene and cDNA for the human high affinity Immunoglobulin E receptor β chain and expression of the complete human receptor. *J. Biol. Chem.* **267**, 12782–12787.
10. Maeker, H. T., Todd, S. C., and Levy, S. (1997) The tetraspanin superfamily: Molecular facilitators. *FASEB J.* **11**, 428–442.
11. Sandford, A. J., Shirakawa, T., Moffatt, M. F., Daniels, S. E., Ra, C., Faux, J. A., Young, R. P., Nakamura, Y., Lathrop, G. M., Cookson, W. O. C. M., and Hopkin, J. M. (1993) Localization of atopy and β subunit of high-affinity IgE receptor (FcεRI) on chromosome 11q. *Lancet* **341**, 332–334.
12. Tedder, T. F., Disteche, C. M., Louie, E., Adler, D. A., Croce, C. M., Schlossman, S. F., and Saito, H. (1989) The gene that encodes the human CD20 (B1) differentiation antigen is located on chromosome 11 near the t(11;14) (q13;q32) translocation site. *J. Immunol.* **142**, 2555–2559